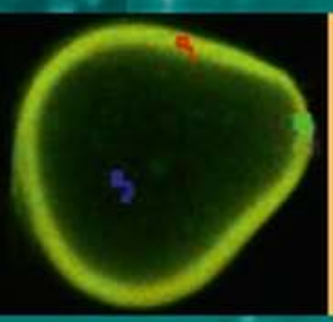
Ajit Varma
Editor



Mycorrhiza

Genetics and Molecular Biology
Eco-Function
Biotechnology
Eco-Physiology
Structure and Systematics

Third Edition

Springer

VISIT…

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Mycorrhiza

State of the Art, Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics

Third Edition

Springer

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Foreword

Terrestrial ecosystems are driven by microbial guildes. We are, however, severely hampered by our limited understanding of the diversity and function of such microbial ecosystems. Growing on mineral particles and decaying organic matter, and living in the vicinity of or within plant roots, are a cast of hundreds of competing versatile and diverse fungal species, including soil decomposers, pathogens, endophytes and obligate symbionts (Frankland 1998). Amongst the extensive intermingling webs of hyphae permeating the soil, those of mycorrhizal fungi play a crucial role in ecosystem sustainability through their role in biogeochemical cycles. These mycorrhizal species are no marginal oddity, having been shown to account for half of the fungal species in most temperate, montane and boreal forests. The hyphae of mycorrhizal fungi and plant short roots form a novel composite organ, the so-called mycorrhiza, which is the site of nutrient and carbon transfer between the two symbiotic partners. This mutualistic interactions allow terrestrial plants to grow efficiently in suboptimal environments (Read and Perez-Moreno 2003). They are key drivers of ecosystem function, receiving 15–25% of carbon net productivity and providing most of the host plant nitrogen and phosphorus. The symbiotic relationship between roots and these fungi is undoubtly one of the most prevalent associations in all terrestrial ecosystems. Knowing which processes these soil fungi are responsible for, and how, is thus increasingly important for understanding the inputs and outputs in forest ecosystems under global change. In this book, many of those at the forefront of the research field integrate and comment on recent developments and ideas on the molecular biology, physiology, and ecology of the mycorrhizal symbioses. All of the major types of mycorrhiza are considered. By taking a broad perspective, they show how new information on mycorrhizal fungi, but also on interactions involving endophytes, nitrogen-fixing bacteria and mycorrhiza helper bacteria, may contribute to concepts and ideas of biology and ecology as a whole. Just as important, they contribute to further invigoration of mycorrhizal research by illuminating the field with new ideas and concepts, derived in part from other fields of plant biology and mycology. Attempts to improve productivity of ecosystems by inoculation with more effective fungal symbionts are also described.

The work described here confirms that the ecological performance of mycorrhizal fungi is a complex phenotype affected by many different traits and by environmental factors. In this Foreword, I will look to future challenges that lie ahead.

Understanding the complexity of the interactions between mycorrhizal symbionts and how this mutualistic association adapts and responds to changes in the biological, chemical and physical properties of the rhizosphere remains a significant challenge for plant and microbial biologists. Identification of the primary determinants controlling the symbiosis development and its metabolic activity (e.g., phosphorus, nitrogen and water acquisition) will open the door to understanding the ecological fitness of the ectomycorrhizal symbiosis. Without any doubt, anatomical features of the mycorrhizal mycelial network (e.g., extension of the extramatrical hyphae) resulting from the symbiosis development is of paramount importance to the metabolic and ecophysiological fitness of the mature mycorrhiza.

In the past decade, research in the field has strongly benefited from approaches aimed at the elucidation of the molecular mechanisms of mycorrhiza developmental processes and key concepts and principles have been elaborated (Section II of this book; see also Harrison 2005; Paszkowski 2006; Martin et al. 2007). The genomics era for mycorrhizas is not yet in full swing, but it is clear from recent studies, highlighted in the reviews in this book, that functional genomics have already made significant contributions to our understanding of developmental and metabolic mechanisms leading to the formation and functioning of mycorrhizas. What will the future bring? We are likely to see imminent advances in understanding of the molecular and cellular mechanisms of the coordinated regulation of developmental and metabolic gene expression in symbiotic partners, although the equally important mechanisms that modulate cell growth rates and shape during mycorrhiza development have not generated a similar intensity of interest. With the genome of the ectomycorrhizal *Laccaria bicolor* in hand (Martin et al. 2008), and genomes of the ectomycorrhizal ascomycete *Tuber melanosporum* and the endomycorrhizal *Glomus intraradices* on the way, we should see rapid progress in elucidating the molecular processes involved in mycorrhizal interactions. The genome sequence of these mycobionts, their analysis, associated genomics and bioinformatics tools provide an unprecedented opportunity to identify the key components of organism–environment interactions that modulate ecosystem responses to global change and increased nutrient input needed for faster growth of biomass feedstock. The immediate benefit of these genomic and genetic resources is the increased precision with which mycorrhizasts can characterize the gene networks controlling the developmental pathways in fungal symbionts. Alternatively, these genomes will facilitate whole-genome transcript profiling for further characterization of the repertoire of symbiosis-regulated genes – from the well-understood protein-coding genes to the more elusive DNA motifs that regulate gene expression. We should be able to answers such questions as: What is the role of rhizospheric chemicals and cellular signals in symbiosis development? How many gene networks control mycorrhiza development, as distinct from providing the housekeeping functions of the fungal and plant cells? What could be the molecular basis of such a progressive, highly organized ontogenic process? The genetic mechanism of symbiosis, which contributes to the delicate ecological balance in healthy forests, also provides insights into plant health that may enable more efficient carbon sequestration and enhanced phytoremediation – using plants to clean up environmental contaminants. We now

have the opportunity to gain fundamental insights into plant development and growth as related to their intimate interaction which symbiotic fungi. These genome-based studies will hopefully spur work on in situ functions of mycorrhizal individuals (genets), populations and communities through ecological genomics.

The aim of ecological genomic studies is to identify the genes and genetic pathways that underlie important ecological responses and interactions, determine the extent to which those genes and pathways exhibit functional variation in nature and characterize the ecological and evolutionary consequences of that variation (Ungerer et al. 2008). Another goal of ecological genomic research is to understand how genomes interact at higher levels of organization, for example, is there a 'community mycorrhizal (meta)genome' and, if so, can we understand how it functions? When considering the mutualistic interaction of mycorrhizal fungi with their hosts and the impact of the environmental factors on the fitness of the symbiosis, we would like to identify the gene networks and gene functions that matter most in a given ecological interaction. The prerequisite of such studies is the acquisition of census of mycorrhizal fungi in their patchy soil environment. During the past decade, PCR-based molecular methods and DNA-sequencing have been routinely used to generate census of mycorrhizal fungi in numerous ecosystems, and the application of these genotyping methods has provided detailed insights into the complexity of mycorrhizal fungal communities and populations, and offers exciting prospects for elucidation of the processes that structure mycorrhizal fungal communities (Horton and Bruns 2001). These tools have managed to reveal the tremendous diversity of mycorrhizal fungi interacting with their host in space and time, but also how different environmental factors and land usage could alter the composition of these soil fungal communities. Applied to species survey, the use of DNA arrays is likely to bring typing of hundreds of individuals, or even entire populations, into the realm of practical reality. If mycorrhizasts follow the tracks of their plant or bacterial geneticist fellows, environmental soil samples will also be probed with hundreds to thousands of different protein-coding gene probes (e.g., GeoChip functional gene arrays; He et al. 2008) to tackle ecological questions such as regulation of nutrient acquisition by extramatrical mycorrhizal hyphae. Single nucleotide polymorphisms (SNPs) and high-density DNA oligoarrays of model mycorrhizal species, such as *L. bicolor*, and its sequenced host tree, *Populus trichocarpa*, usher in the possibility of determining allelic imbalance at hundreds of symbiosis-regulated loci from hundreds of DNA samples, allowing the contemplation of genome-wide association studies (Zhu and Salmeron 2007) to determine the genetic contribution to this complex polygenic processes. A combination of the analysis of community/population composition of mycorrhizal fungi by high-throughput DNA genotyping with in situ assessment of function by transcript profiling will have a substantial effect on the kinds of questions that can be addressed in ecology, particularly for model symbionts where genome sequences and transcriptome have been released (e.g., *Laccaria, Pisolithus, Hebeloma, Glomus, Medicago* and *Populus*). This book is therefore not the last word on mycorrhizal research; it is a description of where we stand, how we got here and perhaps a view of where we might be headed.

Francis Martin

References

Frankland JC (1998) Fungal succession – unravelling the unpredictable. Mycol Res 102:1–15
Harrison M (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42
He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. Int Soc Microb Ecol J 1:67–77
Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871
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Martin F, Aerts A, Ahrén D, Brun A, Danchin EGJ, Duchaussoy F et al. (2008) Symbiosis insights from the genome of the mycorrhizal basidiomycete *Laccaria bicolor*. Nature 452:88–92
Paszkowski U (2006) A journey through signaling in arbuscular mycorrhizal symbioses 2006. New Phytol 172:35–46
Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol 157:475–492
Ungerer MC, Johnson LC, Herman MA (2008) Ecological genomics: understanding gene and genome function in the natural environment. Heredity 100:178–183
Zhu T, Salmeron J (2007) High-definition genome profiling for genetic marker discovery. Trends Plant Sci 12:196–202

Preface

It has been a pleasure to edit this book, primarily due to stimulating discussions with a large number of eminent scientists working on mycorrhizal science and other root endophytes, students and fellow colleagues. The first and second editions were jointly edited with Professor Dr. Berthold Hock, Technical University, München, Germany, and published in 1995 and 1999, respectively. This third edition falls into a time period exceptionally rapid growth in mycorrhizal research. Therefore, the editor has been most pleased with the decision of Springer-Verlag to revise and update and to incorporate the remarkable advances experienced in mycorrhizal field.

A vast expansion of interest in mycorrhiza, resulting in public awareness that the productivity of plants and the quality of leaves, flowers, fruits and seeds are determined by the activities of root systems and their associated physical, chemical and biological environment, is manifest worldwide. Symbiotic fungi have become important subjects of tests to evaluate some of the new opportunities being developed in biotechnology. While these fungi have been used to stabilize eroded soils and the forests since the turn of century, the novelty in recent years has been increased recognition that biological processes can be manipulated genetically, opening up numerous unexplored opportunities for the optimization of plant productivity in both managed and natural ecosystems, while minimizing the risks of environmental damage. The book contains the current state of knowledge and theories on the structure, function, molecular biology and biotechnological applications of mycorrhizas. It will thus be of interest to a diverse audience of researchers and instructors, especially biologists, biochemists, agronomists, foresters, horticulturists, mycologists, soil scientists, ecologists, plant physiologists, microbiologists and landscape architects.

In planning this book, invitations for contributions were extended to leading international authorities working with symbiotic fungi. I would like to express my sincere appreciation to each contributor for his/her work, patience and attention to detail during the entire production process. It is hoped that the reviews, interpretation and concepts proposed by the authors will stimulate further research, as the information presented tends to highlight both the need for further work in this challenging field and the lack of agreement on some fundamental issues.

The encouragement and inspiration received from the Dr. Ashok K Chauhan, Founder President, Ritnand Balved Education Foundation, Sri Atul Chauhan,

Chancellor, Amity University Uttar Pradesh, and Sri Aseem Chauhan, Chancellor, Amity University Rajasthan need special mention. I am indebted to Dr. Dieter Czeschlik for his continued interest and Ursula Gramm for active help. I wish to acknowledge the help and support given to me by my students, faculty colleagues, family members and friends for their constant encouragement.

Amity University
Uttar Pradesh, India
April 2008

Ajit Varma

Preface

Understanding mycorrhizas requires a deep insight into the scope of biology, a discipline with extremely wide boundaries. They reach from the most fundamental genetic and molecular aspects to the diverse facets of ecology. Even biotechnology, the use of biological systems for technological applications, is a firm part of biology. Therefore it is not surprising that the biology of mycorrhiza is subject to the same manifold, which basically reflects the complexity of biological systems.

The third edition of this mycorrhiza book has implemented this insight in an exemplary way. It not only covers the highlights of mycorrhizal interactions, but also makes visible the connections between distant fields of mycorrhiza research, which is most important for future progress. The particular challenge relates to the fact that mycorrhiza deals with the symbiotic association of two most differing systems, plants and fungi. In addition, tremendous progress, achieved during the last ten years, particularly in the fields of genetics and taxonomy, had to be incorporated.

Considering these requirements it is not surprising that all chapters of this edition and therefore the contents are new and up to date. Most of them have been written by new authors of an upcoming generation of mycorrhiza specialists. However, the basic structure of the book has only been slightly changed except for a stronger emphasis on ecological aspects.

Although the emphasis is laid upon arbuscular and ectomycorrhizal associations, it is appreciated that the new edition includes novel root-associated fungal symbionts, which exhibit traits similar to mycorrhizas, as well as *Frankia* and helper bacteria. The fact that beneficial effects as well as interactions with pathogens and parasites are also considered shows that the importance of mutual relations as a general aspect of biology has been kept in mind.

It is hoped that this new edition will not only impart latest results of mycorrhiza research to the interested reader but also encourage young researchers to enter a rewarding as well as challenging field.

Weihenstephan — Bertold Hock
April 2008

Preface to the First Edition

Recent developments in the study of mycorrhizas have encouraged us to present a new book on progress in this field. A vast expansion of interest in mycorrhiza, resulting in public awareness that the productivity of plants and the quality of leaves, flowers, fruits and seeds are determined by the activities of root systems and their associated physical, chemical and biological environment, is manifest worldwide. During its life cycle, a plant root is associated with a myriad of soil microorganisms, especially mycorrhizal fungi. These associations are principally dynamic.

Mycorrhizal fungi have become an important object of tests to evaluate some of the new opportunities being developed in biotechnology. While these fungi have been used to stabilize forests since the turn of century, the novelty in recent years has increased recognition that biological processes can be manipulated genetically, opening numerous opportunities for the optimization of plant productivity in both managed and natural ecosystems, while minimizing the risks of environmental damage. It has become increasingly clear that the vast, expanding field of molecular biology will have a major impact on mycorrhizal studies.

This work summarizes and updates both the state of knowledge and theories on the structure, function, molecular biology and biotechnological applications of mycorrhizas. It will thus be of interest to a diverse audience of researchers and instructors, especially biologists, biochemists, agronomists, foresters, horticulturists, mycologists, soil scientists, ecologists, plant physiologists, microbiologists and landscape architects.

In planning this book, invitations for contributions were extended to leading international authorities working with mycorrhizas. We would like to express our deep appreciation to each contributor for his/her work, patience and attention to detail during the entire production process.

It is hoped that the reviews, interpretations and concepts proposed by the authors will stimulate further research, as the information presented tends to highlight both the need for further work in this field and the lack of agreement on some fundamental issues. There is strong debate, for instance, on the usage of certain terminology such as arbuscular mycorrhizas (AM) or vesicular-arbuscular mycorrhizas (VAM), mycorrhizae or mycorrhizas, and there are divergent opinions on the existence of endomycorrhizins. For the sake of uniformity, the editors had to make some

compromises, but these did not interfere with the different views which often indicate rapidly expanding fields.

We particularly hope that this work will serve as a useful focal point for further studies on the interactions between plants and soil (where fungal hyphae function as the strong bridging link between these two systems) thus providing impetus for the further development of agriculture, horticulture, viticulture and arboriculture which could maintain our potential for food production and simultaneously sustain soil fertility, while avoiding anthropogenic environmental pollution and the waste of energy resources.

It has been a pleasure to edit this book, primarily due to the stimulating cooperation of the contributors. We would like to thank Springer-Verlag and especially Dr. Dieter Czeschlik, for his help and active cooperation during the preparation of this work. We are grateful to Dr. Alexander Hahn for his comments on the manuscript as well as to Mrs. Ingrid Musset and Mrs. Helga Müller for their kind help in preparing the transcripts.

We are confident that the joint efforts of authors and editors will contribute to a better understanding of advances in the study of mycorrhizas and will stimulate further progress.

New Delhi — Ajit Varma
Weihenstephan — Beriold Hock
November 1994

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Mycorrhizal Fungi: What We Know and What Should We Know?

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1 Introduction

Mycorrhizal fungi are symbionts in roots of the majority of higher plants. These associations vary widely in structure and functions, but the most common interaction is the arbuscular mycorrhizal (AM) association. It is estimated that more than 80% of all terrestrial plants form this type of association. These include of many agriculturally and horticulturally important crop species (Smith and Read 1997). The AM symbiosis represents an ancient symbiosis (Pirozynski and Malloch 1975; Pirozynski and Dalpe 1989). Hyphae and arbuscules have been reported in fossils of *Aglaophyton* isolated from the Rhynie chert, and this evidence has established the existence of AM symbiosis in the early Devonian (Remy et al. 1994; Taylor et al. 1995). Furthermore, molecular works based on the nucleotide sequence divergence of 18s rDNA suggests that the Glomales arose 350–460 million years ago and that the symbiosis was instrumental in the successful colonization of land by plants (Simon et al. 1993a). During evolution, the AM symbiosis has been lost from about 10% of plants, including whole angiosperm families (Tester et al. 1987). AM fungi are probably the most ubiquitous fungi in agricultural soils, accounting for 5–36% of the total biomass in soil and 9–55% of the biomass of soil microorganisms (Olson et al. 1999). These fungi are a critical component in agricultural systems because these organisms can increase plant growth (Smith and Read 1997), plant reproductive capacity (Lu and Koide 1994), plant water stress tolerance (Gupta and Kumar 2000), and plant health through antagonistic and competitive effects on pests and pathogens (Gange and West 1994). This colonization may also enhance the plant's resistance to biotic and abiotic stresses (Newsham et al. 1994; Subramanian et al. 1995; von Reichenbach and Schonbeck 1995; Ricken and Hofner 1996). AM fungi develop an extensive hyphal (extraradical hyphae)

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network with the plant root system which makes a significant contribution to the improvement of soil texture and water relations (Bethlenfalvay and Shuepp 1994). The main benefit to the host plant in the mycorrhizae symbiosis is the enhanced uptake of immobile soil nutrients, in particular phosphorus (Jakobsen 1999). Arbuscular mycorrhizal associations increase nitrogen accumulation in plant tissues as a result of the hyphae competing for mineralized organic soil nitrogen (Ibijbijen et al. 1996). Also, these fungi interact with other soil organisms involved in important nutrient cycles. For example, biological nitrogen fixation by *Rhizobium* in legume hosts can be enhanced through co-infection with AMF (Xavier and Germida 2002). Such ecological roles are of special importance in low-input farm management systems because these systems rely on natural nutrient cycles to provide the nutrients required for plant production. Therefore, these fungi constitute an integral and important component of ecosystems and may have significant applications in sustainable agricultural systems (Schreiner and Bethlenfalvay 1995). The process of AM fungal colonization of host plant roots is characterized by distinct stages involving a series of complex morphogenetic changes in the fungus: spore germination, hyphal differentiation, appressorium formation, root penetration, intercellular growth, arbuscule formation, and nutrient transport. For in depth discussions on aspects of the development of the arbuscular mycorrhizal symbiosis, readers are referred to a number of comprehensive reviews (Smith and Gianinazzi-Pearson 1998; Koide and Schreiner 1992; Bonfante and Perotto 1995; Harrison 1997, 1998, 1999; Hirsch and Kapulnik 1988; Albrecht et al. 1999). The AM fungus is a symbiotic organism, but it also harbors structures called bacterium-like organisms (BLOs), which have been detected ultrastructurally in spores and germinating and/or symbiotic mycelium (Bianciotto et al. 1996). These bacteria have been shown to be group II Pseudomonads (genus *Burkholderia*) by DNA sequence analysis of small subunit rRNA genes. However, the significance of these bacteria remains unknown, but they indicate that mycorrhizal systems can include plant, fungal and bacterial cells (Bianciotto et al. 1996, 2000). AM fungi are obligate biotrophs whose completion of their life cycle depends on their ability to colonize a host plant. Furthermore, fungal growth ceases after approximately 25–30 days of culture in the absence of the host plant. Their obligate status, the coenocytic nature of their spores (Becard and Pfeffer 1993) and recombination (having never been demonstrated; Rosendahl and Taylor 1997), are biological aspects of these fungi which have hindered studies on them and on the symbioses they form with plant roots. The latter is particularly important as a mycorrhizal root is the normal state for most plants. These fungi have not been cultured in the absence of the host plant and this has hampered their mass production and utilization within crop systems (Jarstfer and Sylvia 1992). However, it is possible to grow AM fungi in sterile culture with plant root explants (Mosse and Hepper 1975; Miller-Wideman and Watrud 1984; Diop et al. 1994) and/or with hairy roots transformed with *Agrobacterium rhizogenes* (Mugnier and Mosse 1987; Becard and Fortin 1988; Diop et al. 1992; Declerck et al.1996, 1998; Pawlowska et al. 1999). Excised root culture systems have been used to study many aspects of the biology of AM fungi (Balaji et al. 1995; St-Arnaud et al. 1995; Bago et al. 1996; Nagahashi et al. 1996;

Villegas et al. 1996; Douds et al. 1998; Pfeffer et al. 1999). Furthermore, *in vitro* culture provides an abundant source of contaminant-free AM fungal hyphal and spore tissue that is a necessary prerequisite for molecular studies. Association between hosts and these symbionts are usually non-specific (Mosse and Hepper 1975). Most of the evidence for non-specific associations has been demonstrated by inoculating roots with propagules of species of AM fungi in separate pot cultures (Smith and Read 1997). However, it may be possible for a plant grown in field soil to be preferentially colonized by one of the species of the AM fungi present. The establishment of functional symbiosis between AM fungi and host plants involves a sequence of recognition events between the fungus and the plant (Giovanetti et al. 1993; Giovanetti and Sbrana 1998). The understanding of how the AM symbiosis is established and functions is a key issue in plant development. Therefore, understanding the biology of the fungal partner is a cornerstone both of this and of how plant roots function in nature. Since the advent of molecular biology, innovative techniques have been developed which help to overcome some of the problems encountered in the past.

2 Origin and Evolution of Mycorrhizal Studies

Arbuscular mycorrhizas may have been described as early as 1842 (Nageli 1842), but most of Nageli's drawings only remotely resemble the arbuscular mycorrhiza. Frank (1885) gave the name "Mycorrhiza" to the peculiar association between temperate forest tree roots and ectomycorrhizal fungi. In another publication, Frank (1887) recognized a distinction between ectotrophic and endotrophic mycorrhizas, which included at the time only ericaceous and orchid mycorrhizas. Janse (1897) called the intramatrical spores "vesicles" and Gallaud (1905) called the other commonly observed intracellular structures "arbuscules". Thus, the name "vesicular-arbuscular mycorrhiza" was established and persisted until recently. Extensive surveys of host plants and sophisticated anatomical descriptions of what are most certainly arbuscular mycorrhizas are given by Schlicht (1889), Dangeard (1896), Janse (1897), Petri (1903), Gallaud (1905), Peyronel (1924), Jones (1924) and Lohman (1927). Gallaud (1905) made very accurate observations of the arbuscule, and concluded, for example, that it is entirely surrounded by a host membrane, which was later confirmed by Cox and Sanders (1974) using transmission electron microscopy. Gallaud (1905) further distinguished between *Arum* and *Paris* types of arbuscules (Smith and Smith 1997). Light and electron microscopical studies of arbuscular mycorrhizas were facilitated by the founding in 1950 of the Centro di Studio Sulla Micologia del Terreno by Peyronel in Torino, Italy (Bonfante et al. 1991). Scannerini and Bellando (1968) first noted that a space between the host membrane and the fungal wall contained materials of host origin, probably unconsolidated components of host cell wall. Early researchers used classical methods of cutting and staining sections of paraffin-embedded roots to produce excellent drawings and photographs of the arbuscular mycorrhiza. Alternatively, very good

photographs have been obtained by cutting sections of fresh roots on a freezing microtome. However, both methods are laborious if mycorrhization is to be quantified. The problem was solved largely by clearing the roots of cytoplasm by heating in KOH and staining fungal cell walls with Trypan blue in lactophenol (Phillips and Hayman 1970). With the broad application of clearing and staining, however, arbuscular mycorrhizas were more readily documented in abundance in many habitats (Read et al. 1976). Quantification of mycorrhization has been achieved in various ways. Many early studies simply cut root systems into small pieces and determined the proportion of the pieces that were mycorrhized. Probably the most popular method today is based on the line intersect technique devised by Newman (1966), which was possibly first applied to mycorrhizas in 1975 (Sparling and Tinker 1975). Giovannetti and Mosse (1980) later compared various methods for mycorrhiza quantification, which led to greater acceptance of the line intersect method. Trappe (1987) estimated that, in natural communities, approximately 70% of higher plants were obligately dependent upon fungal associates, 12% being facultatively dependent and 18% being typically non-colonized. AM fungi are believed to be disseminated intercontinentally prior to continental drift, as supported by fossil records of earlier plants (Berch 1986; Stubblefield et al. 1987; Remy et al. 1991; Hass et al. 1994). Simon et al. (1993b) have used SSU (small subunit and RNA) sequences as a molecular dock to interdates of divergence between the major clades in the phylogenetic tree of the Glomales. They delineated three families of AM fungi, Acaulosporaceae, Gigasporaceae and Glomaceae. Wu (1993) hypothesized a model of smooth evolutionary transition between relatively unorganized *Glomus*-like sporocarps of *Sclerocystis rubiformis* and intermediate forms like *S. clavispora, S. liquidambaris* and *S. sinousa* to *S. coremioides*. He concluded that *S. coremioides* was not unique. This series of transformations led Wu (1993) to reject the changes of Almeida and Schenck (1990) and revert to the previous classification of Gerdemann and Trappe (1974) who included four species of *Sclerocystis* in their classification scheme of Endogonaceae. Link (1809) established the genus *Endogone*. Tulasne and Tulasne (1844) were the first to describe the genus *Glomus*, known only from spore clusters found in the soil. The Tulasne brothers considered *Glomus* to be closely related to *Endogone*. Fries (1849) established the Endogonaceae, placing it in the Tuberales, but the family was transferred to the Mucorales by Bucholtz (1912). In 1922, Thaxter revised the Endogonaceae, placing the *Glomus* of Tulasne and Tulasne into *Endogone* (Thaxter 1922). He considered *Endogone* to contain both zygosporic (notably *Endogone lactiflua*) and chlamydosporic species, observing that at least one species apparently produced both kinds of spores. In the early 1970s, it became clear to Gerdemann and Trappe (1974) that *Endogone*, which now contained a wide variety of species, needed further revision. They split the old *Endogone sensu lato* into seven genera, including *Endogone, Modicella, Glaziella* (nonmycorrhizal genera), and four mycorrhizal genera, including *Glomus*, a previously described mycorrhizal genus, *Sclerocystis*, and two new genera *Gigaspora* and *Acaulospora*, which corresponded to the honey-colored sessile spores of Mosse and Bowen (1968). These were all placed in the Endogonaceae, Zygomycetes.

Trappe and Schenck (1982) recognized another mycorrhizal genus, *Entrophospora*. In 1987, Walker also recognized five arbuscular mycorrhizal fungal

genera, having dropped *Sclerocystis* and added *Scutellospora*. In 1990, Morton and Benny placed the five genera of Walker (1987) into three families (Glomaceae, Acaulosporaceae, Gigasporaceae) and two suborders (the Glomineae and Gigasporineae), both of which were then placed in a new order, the Glomales. Later, Morton and Benny (2001) recognized two other families, the Archaeosporaceae and Paraglomeraceae, with two new genera, *Archaeospora* and *Paraglomus*. Schuessler et al. (2001) used molecular data to establish the relashionships among arbuscular mycorrhizal fungi and between arbuscular mycorrhizal fungi and other fungi. The group of arbuscular mycorrhizal fungi was elevated to the level of phylum (Glomeromycota), which was shown to be as distinct from other fungi as the Ascomycota are from the Basidiomycota. The Zygomycota were shown to be polyphyletic, and *Endogone* did not group near the Glomeromycota and nor did it group with the Mucorales. *Geosiphon pyriforme* was added to the Glomeromycota, which may have far reaching effects on our understanding of the arbuscular mycorrhizal symbiosis. The methods employed by taxonomists have become increasingly sophisticated. Initially, of course, taxonomies were based upon morphological and anatomical characteristics of the fungi. Later, methods based on serology (Aldwell and Hall 1978), isozyme variation revealed by gel electrophoresis (Hepper 1987), and fatty acid variations (Bentivenga and Morton 1994) were introduced. Systematics of fungi have come to rely increasingly on DNA-based methods (Cummings 1990; Davidson and Geringer 1990; Simon et al. 1990, 1992, 1993b; Redecker 2000). While DNA variation may be the best measure of genealogical relationships among organisms, it is amazing the extent to which anatomical and DNA-based methods have yielded complementary results. Routine identification of arbuscular mycorrhizal fungi will probably continue to be based primarily on structural characters, and there is an increased appreciation that the relationship between anatomy and DNA will be important.

3 Development of AM Fungi

The AM fungi are the most complex group of mycorrhizas which forms interradical structures: (1) intracellular hyphae forming coils, often found in the outer layers of cortical parenchyma, (2) the intercellular hyphae, (3) the intracellular hyphae with numerous ramifications, i.e. the arbuscules, and (4) the inter- or intracellular hypertrophied hyphae, i.e. the vesicles. The mycelial network surrounding the roots is dimorphic: (1) with coarse thick walled irregular non-septate hyphae, and (2) smaller, thin walled ephemeral lateral branches (Mosse 1959a, 1959b; Nicolson 1959, 1967). The thick-walled hyphae penetrate the host root and cause internal infection. At the entry point, the penetrating hyphae form appressoria in the host plants (Gianinazzi-Pearson et al. 1991; Giovannetti et al. 1991, 1993). The penetrating, infecting hyphae spread inter- and intracellularly in the host root cortex. The highly branched structures called arbuscules are usually formed in the inner cortex. These are ephemeral structures formed by the repeated dichotomous branching and complete their development in 4 or 5 days (Brundrett et al. 1985).

Arbuscules are the key sites for nutrient exchange and remain active only for 4–15 days (Carling and Brown 1982; Cox and Tinker 1976). Many but not all endomycorrhizal fungi which form arbuscules later also form terminal or intercalary vesicles in the root cortex. These are expanded; thin-walled structures which are not delimited by a septum but often contain a large quantity of lipids called as vesicles. They may be spherical, oval or lobed and may become thick-walled and resemble resting spores. They serve as the endophytic storage organs and are rich in lipids.

4 Beneficial Effects of AM Fungi

4.1 Nutrient Transport

In the majority of mycorrhizal types, carbohydrates produced by photosynthesis move from the autotroph (host plant) to the heterotroph (fungal symbiont); while nutrients acquired from the soil solution pass in the opposite direction (Smith and Read 1997; Jakobsen 1999). The contribution of AMF to plant nutrient uptake is mainly through the acquisition of nutrients (especially P) from the soil by the extraradical fungal hyphae, especially from root-distant soil not depleted of nutrients by the root (George 2000). Mechanism of nutrient translocation to the host and the carbon drain (photosynthates) of the fungus on the host has significant affects on plant growth (Schellenbaum et al. 1998). Fungal hyphae are functionally analogous to fine root hairs as both are nutrient uptake organs. Diameters of fine root hairs, 5–20 µm (Wulfsohn and Nyengaard 1999), and hyphae, 3–7 µm (Bago 2000; Dodd et al. 2000), are comparable, but hyphal length densities of AMF in soil of chamber and field experiments range from 10- to 100-fold greater than root length densities in the corresponding studies (Miller et al. 1995; Ravnskov et al. 1999; Schweiger et al. 1999; Dodd et al. 2000). Fungal hyphae extend the plant's effective absorption surfaces beyond the nutrient depleted zone that develops around the root caused by direct root uptake processes. However, greater hyphal density is not of equal significance for uptake of all ions in soil (Jakobsen 1999; George 2000). It is of importance for ions with small effective diffusion coefficients (D_e) in soil, such as $H_2PO_4^-$ (10^{-8} to 10^{-11} Cm^2S^{-1}) (Barber 1984). Evidence suggests AMF produce extracellular phosphatase that mineralize organic P for uptake (Joner et al. 2000; Koide and Kabir 2000), but the activity of AMF phosphatase is relatively small in comparison to the activity of other soil microbes and autolysis (Joner et al. 2000).

4.2 Micronutrient Uptake

Micronutrients are needed by the plant in small quantities but are very important for proper growth and development, as they are parts of various enzymes, pigments and other biological molecules essential for plant life. These elements are copper,

zinc, magnesium, manganese and cobalt. AM fungi help the plant in two ways: firstly, they help in the uptake of these elements which are considered to be relatively immobile, and secondly, they take up these elements and store them so as to prevent their concentrations to reach toxic levels. AM fungi could act as a sink for copper, cobalt and zinc (Bowen et al. 1974; Cooper and Tinker 1978). Most benefits of mycorrhiza have been traced to phosphorus uptake. Other elements like zinc also probably play a key role in increase of growth and yield of the plant. Uptake of micronutrients is usually limited by the rate of diffusion, if these elements move through soil to the plant root leading to quick formation of depletion zones around actively growing plant roots. Hyphae of AM fungi extend beyond these zones and help in acquisition and mobilization of these elements (Tinker 1978).

Increased uptake of iron by mycorrhizal fungi may be in part due to production of siderophores that specifically chelate iron. Cress et al. (1979) found siderophore activity associated with four species of AM fungi. A point to be noted here is that iron has also been implicated in ion uptake and metabolism in the *Rhizobium* legume symbiosis.

Elements like potassium nitrate and sulphate in soil solutions have higher mobilities (Nye and Tinker 1977), and it is unlikely that depletion zones would be formed around plant roots and that these nutrients would move more rapidly through hyphae than soil. AM fungi can increase the sulphate-absorbing power of roots, but this appears to be a secondary effect brought about by improved phosphorus nutrition (Rhodes and Gerdemann 1978).

4.3 AM and Nitrogen Fixation

Nitrogen is a non-metallic element needed for formation of amino acids, purines and pyrimidines, and thus indirectly involved in protein and nucleic acid synthesis. It is also a part of porphyrins and many coenzymes of the plant system. Deficiencies of this element lead to spindly growth of the plant and yellowing of leaves. Plants usually take up nitrogen in the form of nitrate, before its incorporation into any of the biological compounds. The process of symbiotic nitrogen fixation carried out by the root nodule bacteria and the ability of many non-symbiotic rhizosphere microbes to fix atmospheric nitrogen are well-recognized aspects of soil microbial activity. Studies have revealed that nodulation by indigenous rhizobia is greatly improved by AM fungi (Hayman 1982). Specific root exudates in mycorrhizal legumes may act as chemotactic attractants to rhizobia (Abbott and Robson 1984). Thus, AM fungal colonization and spread within the root somehow predisposes the legume host to form more nodules resulting in higher nitrogen fixation. AM fungi help in utilization of superphosphate especially in an acid soil. Application of rock phosphate together with AM fungi can improve nodule mass (Mosse 1977) leading to improved nitrogen fixation. Interaction between rock phosphate, AM and symbiotic nitrogen fixation has been studied (Mosse et al. 1976), and it has been shown that legumes inoculated in most phosphorus deficient soils nodulated only in the presence of AM.

4.4 AM and Phytohormones

Hormones accumulation in host tissues is affected by mycorrhizal colonization, with changes in the levels of cytokinin, abscisic acid and gibberlin-like substances (Allen et al. 1980, 1982; Barea 1986). It is unclear if this is linked to improved nutrient status of the host, and it seems unlikely that phytohormone synthesis by the fungus could account for the magnitude of the increase. To what extent altered hormone production in mycorrhizal plants is due to improved nutrient status remains unclear. One AM fungus has been shown to synthesize phytohormones (Barea and Azcon-Aguilar 1982), but whether these can pass into the plant and affect its growth and physiology or its importance in the colonization process has yet to be determined.

4.5 Water Stress

Many studies have reported enhanced survival of mycorrhizal plants over non-mycorrhizal plants under water-stressed situations (Busse and Ellis 1985; Hetrick et al. 1987). One may hypothesize a mechanism of direct uptake and translocation of water via hyphal network similar to the manner of hyphae-mediated nutrient uptake. The likely effect of mycorrhizal colonization on plant drought tolerance is related to nutrient acquisition (Smith and Read 1997). As the soil dries, nutrients become less available because the tortuosity of the diffusion path increases (Barber 1984). It was shown that the higher hyphal length density of mycorrhiza decreases the diffusion distance for nutrients to reach an absorptive surface. Under drought conditions, the contribution of hyphae to nutrient uptake is advantageous to mycorrhizal plants.

4.6 AM and Soil Structure

Soil structure determines characteristics such as water inflow rate, biogeochemical cycling processes, erosion resistance and C storage (Wright and Upadhyaya 1998; Rillig and Steinberg 2002). Soil organic matter plays a major role in aggregation, and organic matter accumulation is a function of biotic activity (Oades 1993; Jastrow 1996). Mechanistically, the role of fungal hyphae and plant roots in soil aggregation can be viewed as a “sticky-string bag” (Miller and Jastrow 2000). The hyphae of AMF entangle and enmesh soil particles to form aggregates in a hierarchal fashion, with the smaller aggregates held together by stronger forces than the larger aggregates (Miller and Jastrow 1990; Oades and Waters 1991). The glycoprotein glomalin is secreted onto hyphal surfaces in copious amounts (Rillig et al. 2001). In terms of fungal physiology, glomalin is a recalcitrant hydrophobic

molecule that enables aerial growth beyond the gas-water interface (Miller and Jastrow 2000). Its concentration in soil has a strong correlation to water stability of aggregates (Wright and Upadhyaya 1998). The hydrophobicity of this molecule may reduce macro aggregate disruption during wetting and drying cycles by retarding water movement into the pores, thereby allowing the non-disruptive escape of displaced gases from the pores (Miller and Jastrow 2000). Rillig and Steinberg (2002) hypothesized that glomalin production is a mechanism of habitat modification by AMF to generate more favorable growth space.

4.7 AM in Natural Ecosystems

Natural ecosystems are disturbed habitats. Here, the diversity of the species (AM) is maintained on the basis of the natural phenomenon of ecosystem survival of the fittest. Mycorrhizal fungi can, to an extent, regulate the communities in which they occur. Today, the biosphere is in danger in two senses: directly through outright destruction for productive use, and indirectly through the burden of waste disposal. Therefore, there is a need to protect ecosystems. Mycorrhizae are believed to protect the environmental quality by enhancing beneficial biological interactions.

A matured ecosystem is characterized by a nutrient conservative system in which nutrient is rapidly cycled between the biotic parts and is not available to leach out of the system. Mycorrhizae are critical in that these fungi permeate the soil, picking up nutrients and channeling them to the host plant. Mycorrhizae regulate the composition and functioning of plant communities by regulating the resource allocation and growth characteristics of interacting plants (Allen 1991). Nicolson (1960) examined the role of mycorrhizal fungi in sand dune succession and found that the highest colonization of the host plant occurred within open communities, particularly within fixed, non-mobile dunes, and the colonization increased as the communities became closed.

Dodd et al. (1990) studied the management of populations of AM fungi in acid-infertile soils of Savannah ecosystem and concluded that different plant hosts can caused the build-up of different populations of AM fungi in the soil around the root system. This indicates that, although species of AM fungi may be effective on a wide range of plants when introduced individually into sterile soil (Howeler et al. 1987), under natural soil conditions, different plants are likely to become infected by several different AM fungi.

4.8 AM in Forestation of Arid Lands

Arid regions comprise approximately one-fifth of the earth's land area and contain a large fraction of the known energy and mineral reserves. Restoration of forest land devastated for resource extraction is an immediate priority and a challenging

task for arid land ecologists. In such areas, to cope with stress situations, plants have developed a number of strategies such as: (1) changes in root absorption capacity, (2) modification of root to shoot ratio, and (3) rhizosphere interactions, etc. AM plays a role in all these adaptations. AM fungi are widespread in forest trees and this symbiosis can be manipulated to enhance productivity in forestation programs. The AM fungi change the supply of mineral nutrients from soil thereby modifying soil fertility, mycorrhizosphere and aggregation of soil particles (Varma 1995). AM actually increases the growth rate of plants and influences the partitioning of phytomass between the root and shoot. The root/shoot ratio is usually lower in AM plants than in their non-mycorrhizal counterparts (Smith et al. 1986). The AM inoculated plants are not only large but also usually have an increased concentration and/or content of phosphorus. The enhancement of growth is primarily a result of increased mineral nutrition of plants. The non-nutritional effects of mycorrhizas in reducing the severity of some plants diseases, and in modifying water relations and soil structure are also potentially important. The benefits of AM may also extend to alleviation of effects of mineral excesses (Allen 1992).

4.9 AM in Establishment of Underutilized Plant Species

Underutilized plants constitute plant species that occur as life support species in extreme environmental situations or threatened habitats, having tolerant genetic make-up to survive in such situations, and also having promising nutritional and industrial importance for a variety of purposes for humankind. Their cultivation is restricted to specialized geographical pockets in different agro-ecological regions and mainly by poor farming communities. Since thee majority of these crop species are grown under rain-fed situations with low inputs in tropical countries like India, it is highly essential to select and introduce arbuscular mycorrhizal fungi which can help in improving plant growth under adverse soil conditions. The ability of AM fungi to overcome stress is well known. In arid regions, where lack of water restricts the production of crops, AM fungi alter hormonal and physiological mechanisms of host plants and control loss of water (Graham and Menge 1982). AM fungi also influence photosynthesis, photorespiration and photosynthetic pigments under drought conditions.

5 Mycorrhiza as Biological Agents

Mycorrhizae interact with plant-associated bacteria, fungi and other organisms and influence the interactions between the host and associated microorganisms (Azcon-Aguilar and Barea 1992; Fitter and Sanders 1992). If the association between the host and the collective AMF community was not compatible, the association can lead to serious losses in crop yields (Hetrick 1984). In contrast, a compatible association results in enhanced plant productivity, through enhanced P nutrition

(Ravnskov and Jakobsen 1995), prevention or control of plant diseases caused by soil-borne pathogens (Caron 1989; St-Arnaud et al. 1995), and enhancement of phytohormonal activity (Frankenberger and Arshad 1995). The augmentation of soil with non-indigenous (elite) strains of microorganisms is promising. However, the performance of these microorganisms depends on the particular soil–plant–environment conditions. Exploitation of indigenous AMF communities to counteract the negative effects of soil-borne plant pathogens only requires that the AMF community be active. An active and diverse AMF community may be able to effectively counteract the challenges of plant pathogens by enhancing the vigor of the plant. The effect of mycorrhizal fungi on bacteria can be broadly classified into three groups: natural (Otto and Winkler 1995), antagonistic (Shalaby and Hanna 1998; Filion et al. 1999) and synergistic (Gryndler and Hrselova 1998; Edwards et al. 1998). Interactions between mycorrhizal fungi and variety of bacteria, such as diazotrophs, biocontrol agents, and other rhizosphere bacteria, often lead to significant improvements in plant growth, yield and nutrition. In other cases, interactions between mycorrhizal fungi and bacteria have resulted in a natural response.

Shalaby and Hanna (1998) examined the interactions between *Glomus mosseae* and *Pseudomonas syringae* in soybean plants and found that *G. mosseae* prevented the infection of soybean plants by *P. syringae*. Li et al. (1997) also found that *G. macrocarpum* reduced the infection caused by *P. lacrymans* in eggplant and cucumber, although no positive growth or yield effect was noted, indicating tolerance to the pathogen as a possible mode of action. Sharma et al. (1995) reported that inoculation of mulberry with *G. fasciculatum* or *G. mosseae* in combination with 60–90 kg of P per hectare per year reduced the incidence of bacterial blight caused by *P. syringae* pv. *mori*. One of the first reports describing biological control of a plant pathogenic fungus using AMF was by Safir (1968). Since then, several workers have successfully demonstrated the potential of mycorrhizal fungi to control plant pathogenic fungi (Duchesne et al. 1989; Kapoor and Mukerji 1998; Kegler and Gottwald 1998; Becker et al. 1999). Successful disease control is reported to range from suppression or elimination of disease to disease tolerance by the host plant. However, the majority of these reports only note whether the disease symptoms are alleviated by the mycorrhizal fungi treatment. Several studies investigating the potential of AMF for biological control of *Fusarium oxysporum f.sp. lycopersici* have been reported (Caron et al. 1985, 1986a, 1986b, 1986c, 1986d).

Significant reductions in wilting by the pathogen were attributed, in part, to increased lignin deposition in the plant cell walls as a result of mycorrhizal colonization, which may have restricted the spread of the pathogen (Dehne and Schoenbeck 1979). Boyetchko and Tewari (1988) reported for the first time the suppression of common root rot, caused by *Bipolaris sorokiniana*, using *Glomus dimorphicum, G. mosseae* and *G. intraradices*, but *G. dimorphicum* was not as effective as the other AMF species tested. Sreeramula et al. (1998) reported that the combined application of *G. fasciculatum* and *T. harzianum* was very effective at controlling damping-off caused by *Pythium aphanidermatum*, and black shank disease caused by *Phytophthora parasitica var. nicotianae* in tobacco seedlings. Kulkarni et al. (1997) reported that the addition of *G. fasciculatum, Gigaspora margarita, Acaulospora laevis* and *Sclerocystis dussii* significantly nullified the destructive

effect of the *Sclerotium rolfsii* on ground nut. Feldmann and Boyle (1998) noted an inverse relationship between *G. etunicatum* colonization of the begonia cultivars and the intensity of powdery mildew caused by *Oidium begoniae*. Using an in vitro system, Filion et al. (1999) demonstrated that extracts from the extraradical mycelium of *G. intraradices* reduced the conidial germination of *F. oxysporum* f.sp. *chrysanthemi*. They also noted that this biological effect was directly proportional to the extract concentration, indicating that compounds released from the external mycelium can control pathogen survival and activity. Alternatively, this indicates that extracts from the extraradical mycelium attend the equilibrium in the mycorrhizosphere in such a manner as to result in inhibition of the pathogen. Weeds are major problems in agricultural systems causing yield reductions and diminishing the quality of crops. Herbicides are still considered the best means of weed control (Sances and Ingham 1997). Some workers have identified the potential of mycorrhizal fungi as agents for the inhibition or reduction of grass weeds such as *Poa annua* and *Lolium perenne*. In the majority of instances of weed inhibition using mycorrhizae, the competing crop or non-weed plant was either inoculated with an AMF species or plant roots were colonized by AMF propagules that were abundant in soil. The underlying implication is that the AMF species may have been highly compatible with the non-weed plant species compared to the weed species, and the AMF propagule number was high. Alternatively, the AMF diversity was high, resulting in a superior AMF assemblage around the roots of the plants and greater competitiveness to the crops. It is unclear whether this bioherbicidal activity is due to competition, production of deleterious substances or physiological/biochemical alterations within the weed plant. The effectiveness of mycorrhizal fungi as biocontrol agents can be improved by consideration of the factors that influence their efficacy, competitiveness and survival. One of the most important factors that may affect the functioning of naturally occurring mycorrhizal fungi in the field, in the context of biological control of pathogens, is the diversity of mycorrhizal fungi at a given site. Mycorrhizal functioning and benefits are generally influenced by the host type and crop rotation (Johnson et al. 1992; Bever et al. 1996), fertilizer application (Vivekanandan and Fixen 1991; McGonigle and Miller 1993), pesticide application (Schreiner and Bethlenfalvay 1997) and the effect of associated microorganisms (Andrade et al. 1995). There are additional factors that can influence the biocontrol activities of mycorrhizal fungi, especially in agro-ecosystems. Some of these factors include mass production for field application, inoculation technology and the competitiveness of the elite mycorrhizal fungi.

6 Factors Affecting the Symbiosis

6.1 Biotic Factors

The influence of the host genetic factor is very significant in initiating the AM to colonize the root. The structure and morphology of roots also play a decisive role in mycorrhization. The microflora in soil and around the roots also influences the formation of AM.

6.1.1 Host Genotype and the Type of Fungus

While the VA fungi are known to vary in their ability to colonize and transfer P to the plant and confer other beneficial effects, very little is known about the exact role of the host genotype in the expression of AM. The efficiency of the same VA fungus can vary very markedly between different species of host plant, so that certain host fungus associations are more effective that others (Lambert et al. 1980). Response to a VA mycorrhiza can also vary within a plant species and cultivar irrespective of infection levels (Hall 1978). The specific cultivar–fungus response may be dependent on soil pH (Skipper and Smith 1979). Menge et al. (1978) attributed these variations in mycorrhizal dependency to the differing ability of plants to absorb phosphorus from low P soils, but other characteristics inherent to plants may also be determinant which include both physiological and anatomical features. Plant species or cultivars which are highly P-dependent tend to be strongly susceptible to AM. Root anatomy is also said to influence mycorrhizal colonization. According to Baylis (1975), mycotrophy is largely a feature of woody to herbaceous plants lacking root hairs (Magnolioid roots). Mycorrhizal colonization shows a significant correlation with this type of root anatomy (Janos 1975).

6.1.2 Interaction with Free-living N_2-fixing Microorganisms

There are reports of positive interaction between free-living N_2-fixing bacteria and AM associations (Bagyaraj and Menge 1978; Fitter and Garbays 1994). AM colonization favorably affects bacterial populations of *Azotobacter* in the rhizosphere of the plant they colonize (Bagyaraj and Menge 1978). Growth stimulation of plants is better under inoculations with both than either microbe alone.

6.1.3 Interaction with Symbiotic Actinomycetes–*Frankia*

Many AM fungi have been observed in close association with different non-legume nitrogen fixing plant species. Preliminary experiments in case of *Casuarina* sp. have shown that double inoculation with AM and *Frankia* significantly improved plant growth and nodulation. Rose and Youngberg (1981) have also reported an increase in plant dry weight, number and weight of nodules and N and P content in dual inoculated plants as compared to those inoculated with actinomycete alone.

6.1.4 Interaction with Phytohormone-producing Bacteria

Phytohormones synthesis by certain bacteria like *Azotobacter, Rhizobium, Pseudomonas* can significantly increase AM colonization (Azcon et al. 1978). Gunze and Hennessy (1980) suggested that application of IAA could influence

arbuscule formation in AM. A large proportion of rhizosphere bacteria are able to produce phytohormones, however, though how and to what extent they affect AM colonization needs to be investigated further.

6.2 Abiotic Factors

Climatic and physico-chemical features of soil in which the host plant is growing influence AM development and establishment.

6.2.1 Light

The mycosymbiont obtain their energy source from the plant and hence rely on both the photosynthetic ability of the plant and the translocation of photosynthates to the root. For such systems, light is understandably a limiting factor. Light has been shown to stimulate development of AM (Furlan and Fortin 1977). Shading not only reduces root colonization and spore production but also the plant response to the mycorrhiza, probably because of reduced spread of internal hyphae within root tissues and a consequent restricted growth of extrametrical hyphae in the soil.

6.2.2 Temperature

Soil temperature also influences all three stages of mycorrhizal development, i.e. spore germination, hyphal penetration of root and proliferation within the cortical cells of root. For spore germination, there is an optimum temperature which varies with the fungal species. Fungal penetration and development in roots is also sensitive to variations in soil temperature (Schenck et al. 1975). Survival of fungal spores on soil after the death or harvest of the host plant is also dependent upon soil temperature. It is thought that its effect may increase or decrease depending upon the texture of the soil (Bowen 1980).

6.2.3 Soil pH

The endophyte efficiency is determined by AM adaptation to soil pH. Soil pH affects both spore germination as well as its development (Daniels and Trape 1980; Angle and Heckmen 1986). Hayman and Mosse (1971) obtained colonization and growth stimulation in two soils of pH 5.6 and 7 but not in acid soils of pH 3.3–4.4. The relationship between soil pH and mycorrhizal effect is complex and depends on the plant species and also on soil type, forms of phosphorus and fungal species involved.

6.2.4 Salinity

There are few studies dealing with AM effects on plant growth under saline conditions (Rosendahl and Rosendahl 1991) or on the effects of salinity on mycorrhizal colonization (Abbott and Robson 1991). AM fungi may have the ability to protect plants from salt stress, but the mechanism is not very clear. In general, there is a decline in propagule production and AM colonization under high salinity conditions (Kim and Webber 1985; Pfeffer and Bloss 1988). However, the little data available do indicate that AM fungi have a potential to enhance the benefits derived from the salt-tolerant crop species if plant–endophyte combinations are properly selected.

7 Mycorrhizal Host Specificity and Seasonality

Arbuscular mycorrhizal fungi are considered to have low specificities of association with host species, but this conclusion is mostly based on experiments in which individual isolates of fungal species are grown separately, apart from competitive interactions (Bever et al. 2001). When fungi are examined as a community, evidence suggests fungal growth rates are highly host-specific. In an experiment in which AMF were trapped on different plant hosts, isolates of different fungal species sporulated differentially, with the relative dominance of fungal species being reversed, depending on the plant species with which they were associated (Bever et al. 1996). As this pattern of host specificity of growth rates in this "non-specific" association has been observed in other systems, including tall grass prairie (Johnson et al. 1992), California grasslands (Nelson and Allen 1993), chalk grasslands (Fitter and Sanders 1992) and agricultural fields (Douds and Millner 1999), this appears to be a general property of this interaction. This specificity of fungal response could contribute to the maintenance of diversity within the AMF community (Bever et al. 2001). Evidence showed that fungal spore density differs seasonally, with some fungi sporulating in late spring and others sporulating at the end of summer. As the spores represent the dormant state of the fungus, the physiologically active state is most likely the mirror image of the seasonal spore counts. For example, *Gigaspora gigantea*, which sporulates most abundantly in the fall and appears over winter as spores, is likely to be physiologically active during the warm season. McGonigle and Fitter (1990) concluded that ecological specificity applied to some arbuscular mycorrhizal associations. However, the competitive balance between AM fungal species in terms of their ability to colonize roots may be affected by environmental conditions. The symbiotic performance of AM fungi and the final mycorrhizal phenotype can be considered as resulting from the interaction of two main factors: infectivity and effectivity, both of which depend on fungal and plant determinants. Although plant mechanisms regulating mycorrhizal infection is complex, it is interesting that plant resistance to arbuscular mycorrhizal fungi can be induced by a simple mutation at one locus (Gollottee et al. 1993).

8 Ecological Significance of AMF Diversity: Inter- and Intraspecies Variations

The number of AMF species in a field community provides only a cursory look at the true level of ecological diversity present at a site. Within populations of a fungal species, or a single spore of the species, there is evidence of abundant genetic variation, in spite of the asexual nature of these organisms (Sanders et al. 1995; Clapp et al. 1999; Jansa et al. 2002b). Sanders et al. (1995) found a different ITS sequence in each of 10 morphologically identical *Glomus mosseae* spores. Clapp et al. (1999) and Jansa et al. (2002a) found several distinct 18S and ITS sequences within a single spore of a *Scutellospora* sp. and *Glomus intraradices*, respectively. It is difficult to draw direct links to the genetics of AMF from the study of ribosomal genes because of their multicopy nature and possibly different evolutionary processes from other genes (Jansa et al. 2002b). However, it became clear that the genetic heterogeneity of nuclei within AMF spores exists also for single copy locus such as the gene encoding the BiP protein (Kuhn et al. 2001). It is not clear whether intra-individual genetic diversity of nuclei in the AMF plays a role in their physiology and ecological tolerance. The observed diversity could be evidence of an adaptive mechanism that allows symbiosis with different plants in a whole range of environments (Van der Heijden et al. 1998; Kuhn et al. 2001; Jansa et al. 2002a, 2002b).

References

Abbott LK, Robson AD (1984) The effect of mycorrhiza on plant growth. In: Powell CL, Bagyaraj DJ (eds) VA mycorrhiza. CRC Press, Boca Raton, Fla, pp 113–130

Abbott LK, Robson AD (1991) Factors influencing the occurrence of vesicular mycorrhizas. Agric Ecosyst Envir 35: 121–150

Albrecht C, Geurts R, Bisseling T (1999) Legume nodulation and mycorrhizae formation two extremes in host specificity meet. EMBO J 18:281–289

Aldwell FEB, Hall IR (1987) A review of serological techniques for the identification of mycorrhizal fungi. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. Practical applications and research priorities. Proceedings of the 7th North American Sciences, University of Florida, Gainesville, Fla., pp 305–307

Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge

Allen MF (1992) Mycorrhizal functioning: an integrative plant–fungal process. Chapman and Hall, New York

Allen MF, Moore TS, Christensen M (1980) Phytochromone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae I. Cytokinin increase in host plant. Can J Bot 91:437–446

Allen MF, Moore TS, Christensen M (1982) Phytochromone changes in *Bouteloua gracilis* infected by vesicular-arbuscular mycorrhizae II. Altered levels of gibberellin-like substances and abscisic acid in the host plant. Can J Bot 60:468–471

Almeida RT, Schenck NC (1990) A revision of the genus *Sclerocystis* (Glomaceae, Glomales). Mycologia 82:703–714

Andrade G, Azcon R, Bethlenfalvay GJ (1995) A rhizobacterium modifies plant and soil responses to the mycorrhizal fungus *Glomus mosseae*. Appl Soil Ecol 2:195–202

Angle JS, Heckman JR (1986) Effect of soil pH and sewage sludge on VA mycorrhizal infection of soybeans. Plant Soil 93:437–441

Azcon R, Azcon G, De Aguilar C, Barea JM (1978) Effects of plant hormones present in bacterial cultures on the formation and responses to VA endomycorrhiza. New Phytol 80:359–364

Azcon-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen MF (ed) Mycorrhizal functioning: an integrative plant–fungal process. Chapman and Hall, New York, pp 163–198

Bago B (2000) Putative sites for nutrient uptake in arbuscular mycorrhizal fungi. Plant Soil 226:263–274

Bago B, Vierheilig H, Piche Y, Azcon-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:223–280

Bagyaraj DJ, Menge JA (1978) Interaction between a VA Mycorrhiza and *Azotobacter* and their effects on rhizosphere mycoflora and plant growth. New Phytol 80:567–573

Balaji B, Poulin MJ, Vierheilig H, Piche Y (1995) Responses of an arbuscular mycorrhizal fungus *Gigaspora margarita* to roots of non-mycorrhizal and mycorrhizal mutants of *Pisum sativum* L. Sparkle. Exp Mycol 19:275–283

Barber SA (1984) Soil nutrient bioavailability: a mechanistic approach. Wiley, New York

Barea JM (1986) Importance of hormones and root exudates in mycorrhizal phenomena. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 177–187

Barea JM, Azcon-Aguilar C (1982) Production of plant growth regulating substances by the vesicular arbuscular mycorrhizal fungus *Glomus mosseae*. Appl Environ Microbiol 43:810–913

Baylis GTS (1975) The magnolioid mycorrhizal and mycotrophy in root systems derived from it. In: Sander FE, Mosse B, Tinker PB (eds) Endomycorrhizas. Academic, London, pp 373–389

Becard G, Fortin JA (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Becard G, Pfeffer PE (1993) Status of nuclear division in arbuscular mycorrhizal fungi *in vitro* development. Protoplasma 174:62–68

Becker DM, Bagley ST, Podila GK (1999) Effects of mycorrhizal-associated streptomycetes on growth of *Laccaria bicolor, Cenococcum geophilum,* and *Armillaria* species and on gene expression in *Laccaria bicolor*. Mycologia 91:33–40

Bentivenga S P and Morton J B (1994) Stability and heritability of fatty acid methyl ester profiles of glomalean endomycorrhizal fungi. Mycol Res 98:1419–1426.

Berch SM (1986) Endogonaceae: Taxonomy, specificity fossil record, phylogeny. In*:* Mukerji KG, Pathak NC, Singh VP (eds) Frontiers in applied microbiology, vol 2. India Print House, Lucknow, pp. 161–186

Bethlenfalvay GJ, Schuepp H (1994) Arbuscular mycorrhizae and agrosystem stability. In: Gianinazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizae on sustainable agriculture and natural ecosystems. Birkhause, Basel, pp 117–131

Bever JD, Morton JB, Antonovics J, Schults PA (1996) Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. J Ecol 84:71–82

Bever JD, Schultz PA, Pringle A, Morton JB (2001) Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. Bioscience 51:923–931

Bianciotto V, Bandi C, Minerdi D, Sironi M, Volker-Tichy H, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itself harbours obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bianciotto V, Lumini E, Lamfranco L, Minerdi O, Bonfante P, Perotto S (2000) Detection and identification of bacterial endosymbionts in arbuscular mycorrhizal fungi belonging to the family Gigasporaceae. Appl Environ Microbiol 46:4503–4509

Bonfante P, Perotto S (1995) Strategies of arbuscular mycorrhizal fungi when infecting host plants. New Phytol 130:3–21

Bonfante-Fasolo P, Tamagnone L, Peretto R, Esquerr-Tugaye MT, Mazau D, Mosiniak M, Vian B (1991) Immunocytochemical location of hydroxyproline rich glycoproteins at the interface between a mycorrhizal fungus and its host planta. Protoplasma 165:127–138

Bowen GD (1980) Mycorrhizal roles in tropical plants and ecosystem. In: Mikola P (ed) Tropical mycorrhiza research. Clarendon Press, Oxford, pp 165–190

Boyetchko SM, Tewari JP (1988) The effect of VA mycorrhizal fungi on infection by *Bipolaris sorokiniana* in barley. Can J Plant Pathol 10:361 (Abstr)

Brundrett MC, Piche Y, Peterson RL(1985) A developmental study of the early stages in vesicular arbuscular mycorrhiza formation. Can J Bot 63:194

Bucholtz F (1912) Beitrage zur Kenntnis der Gattung *Endogone* Link. Beih Bot Zbl 29:147–225

Busse MD, Ellis JR (1985) Vesicular-arbuscular mycorrhizal (*Glomus fasciculatum*) influence on soybean drought tolerance in high phosphorus soil. Can J Bot 63:2290–2294

Carling DE, Brown MF (1982) Anatomy and physiology of vesicular-arbuscular and nonmycorrhizal roots. Phytopathology 72:1108–1114

Caron M (1989) Potential use of mycorrhizae in control of soil-borne diseases Can J Plant Pathol 11:177–179

Caron M, Fortin JA, Richard C (1985) Influence of substrate on the interaction of *Gloums intraradices* and *Fusariun oxysporum* f. sp. *radicislcopersici* on tomatoes. Plant Soil 87:233–239

Caron M, Fortin JA, Richard C (1986a) Effect of *Glomus intraradices* on the infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomatoes over a 12-week period. Can J Bot 64:552–556

Caron M, Fortin JA, Richard C (1986b) Effect of phosphorus concentration and *Glomus intraradices* on *Fusarium* crown and root rot of tomatoes. Phytopathology 76:942–946

Caron M, Fortin JA, Richard C (1986c) Effect of preinfestation of the soil by a vesicular-arbuscular mycorrhizal fungus, *Gloums interaradices,* on *Fusarium* crown and root rot of tomatoes. Phytoprotection 67:15–19

Caron M, Fortin JA, Richard C (1986d) Effect of inoculation sequence on the interaction between *Glomus intraradices* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomatoes. Can J Plant Pathol 8:12–16

Clapp JP, Fitter AH, Young JPW (1999) Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, *Scutellospora* sp. Mol Ecol 8:915–921

Cooper KM, Tinker PB (1978) Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas. IV. Effect of environmental variables on movement of phosphorus. New Phytol 88:327–339

Cox G, Tinker PB (1976) Translocation and transfer of nutrients in vesicular arbuscular mycorrhizae, I. The arbuscule and phosphorus transfer: a quantitative ultrastructural study. New Phytol 77:371–378

Cox GC, Sanders FET (1974) Ultrastructure of the host-fungus interface in a vesicular-arbuscular mycorrhiza. New Phytol 73:901–912

Cress WA, Throneberry GO and Lindsey DL (1979) Kinetics of phosphorus absorption by mycorrhizal and non mycorrhizal tomato roots. Plant Physiol 64:484–487

Cummings BA (1990) Use of RFLPs as a means of examining genetic relatedness in AM fungi. In: Allen MF, Williams SE (eds) Abstracts, 8th North American Conference on Mycorrhizae, University of Wyoming Agricultural Experiment Station, Laramie, Wyo., p 63

Dangeard PA (1896) Une maladie du peuplier dans l'ouest de la France. Botaniste 58:38–43

Daniels BA, Trape JM (1980) Factors affecting spore germination of the vesicular–arbuscular mycorrhizal fungus, *Glomus epigaeus*. Mycologia 72:457–471

Davidson K, Geringer JE (1990) Genetic studies of vesicular-arbuscular mycorrhizal fungi. *In:* Allen MF, Williams SE (eds) Abstracts, 8th North American Conference on Mycorrhizae. University of Wyoming Agricultural Experiment Station, Laramie, Wyo., p 70

Declerck S, Strullu DG, Plenchette C (1996) *In vitro* mass production of the arbuscular mycorrhizal fungus, *Glomus versiforme*, associated with Ri T-DNA transformed carrot roots. Mycol Res 100:1237–1242

Declerck S, Strullu DG, Plenchette C (1998) Monoxenic culture of the intraradical forms of *Glomus* sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. Mycologia 90:579–585

Dehne HW, Schonbeck F (1979) Investigations on the influence of endotrophic mycorrhiza on plant diseases I. Colonization of tomato plants by *Fusarium oxysporum* f.sp. *Lycopersici*. Phytopathol Z 95:105–110

Diop TA, Becard G, Piche Y (1992) Long-term *in vitro* culture of an endomycorrhizal fungus *Gigaspora margarita* on Ri T- DNA transformed roost of carrot. Symbiosis 12:249–259

Diop TA, Plenchette C, Strullu DG (1994) Dual axenic culture of sheared root inocula of vesicular arbuscular mycorrhizal fungi associated with tomato roots. Mycorrhiza 5:17–22

Dodd JC, Arias I, Koomen I, Hayman DS (1990) The management of populations of vesicular-arbuscular mycorrhizal fungi in acid-infertile soils of a savannah AM fungi. Plant Soil 122:241–247

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

Douds DD, Millner P (1999) Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. Agric Ecosyst Environ 74:77–93

Douds DD, Galvez L, Becard G, Kalpulnik Y (1998) Regulation of arbuscular mycorrhizal development by plant host and fungus species in alfalfa. New Phytol 138:27–35

Duchesne LC, Peterson RL, Ellis BE (1989) The time-course of disease suppression and antibiosis by the ectomycorrhizal fungus *Paxillus involutus*. New Phytol 111:693–698

Edwards SG, Young JPW, Fitter AH (1998) Interactions between *Pseudomonas fluorescens* biocontrol agents and *Glomus mosseae,* an arbuscular mycorrhizal fungus, within the rhizosphere. FEMS Microbiol Lett 166:297–303

Feldmann F, Boyle C (1998) Concurrent development of arbuscular mycorrhizal colonization and powdery mildew infection on three *Begonia hiemalis* cultivars. Z Pflanzenkr Pflanzenschutz 105:121–129

Filion M, St-Arnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:252–533

Fitter AH, Garbays J (1994) Interactions between mycorrhizal fungi and other soil organisms. Plant Soil 159:123–132

Fitter AH, Sanders IR (1992) Interactions with the soil fauna. In: Allen MF (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, New York, pp 333–354

Frank AB (1885) Ueber die auf Wurzelsymbiose beruhende Ernahrung gewisser Baume durch unterirdische Pilze. Ber Dtsch Bot Ges 3:128–145

Frank AB (1887) Ueber neue Mycorrhiza-formen. Ber Dtsch Bot Ges 5:395–409

Frankenberger WT, Arshad M (1995) Microbial biosynthesis of auxins. In: Frankenberger WT, Arshad M (eds) Phytohormones in soil. Dekker, New York, pp 35–71

Fries EM (1849) Summa Vegetabilium Scandinaveae 2:261–572

Furlan V (1993) Large scale application of endomycorrhizal fungi and technology transfer to the farmer. In: Peterson L, Schelkle M (eds) Proceedings 9th North American Conference on Mycorrhizae. August 8–12, 1993, Guelph, Ontario, Canada, p 77 (Abstr)

Furlan V, Fortin JV (1977) Effects of light intensity on the formation of vesicular-arbucular endomycorrhizae on *Alluim cepa by Gigaspora calospora*. New Phytol 79:335–340

Gallaud J (1905) Etude sur les mycorrhizes endotrophes. Rev Gen Bot 17:5–48, 66–86, 123–136, 223–249, 313–325, 425–433, 479–500

Gange AC, West HM (1994) Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. New Phytol 128:79–87

George E (2000) Nutrient uptake. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 307–343

Gerdemann JW, Trappe JM (1974) Endogonaceae in the pacific Northwest. Mycol Mem 5:1–76

Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith FA (1991) Enzymatic studies on the metabolism of vesicular arbuscular mycorrhizae V, IsH+-ATP-hydroloysing enzyme a component of ATP-hydrolysing activities in plant-fungus interfaces. New Phytol 117:61–74

Giovanetti M, Sbrana C (1998) Meeting a non- host: the behaviour of AM Fungi. Mycorrhiza 8:123–130

Giovanetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential hyphal morphogensis in arbuscular mycorrhizal fungi during preinfection stages. New Phytol 125:587–593

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Giovannetti M, Sbrana C, Avio L, Citernesi AS (1991) Appressoriun Formation in AM fungi in presence of host and non-host plants. (Abstr) Fungal Cell Biology Ultrastructure, April 1991, Portsmouth, UK

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential morphogenesis in arbuscular mycorrhizal fungi during preinfection stages, New Phytol 125:587–593

Gollottee A, Gianinazzi-Pearson V, Giovannetti M. Sbrana C, Avio L, Gianinazzi S (1993) Cellular localization and cytochemical probing of resistance reactions to rabuscular mycorrhizal fungi in a locus a myc-mutant of *Pisum sativum* L. Planta 191:112–122

Graham JH, Menge JA (1982) Influence of vesicular arbuscular mycorrhizae and soil phosphorus on take-all disease of wheat. Phytopathology 72:95–98

Gryndler M, Hrselova H (1998) Effect of diazotorphic bacteria isolated from a mycelium of arbuscular mycorrhizal fungi on colonization of maize roots by *Glomus fistulosum*. Biol Plant 41:617–621

Gunze CMB, Henessy CMR (1980) Effect of host applied auxin in development of endomycorrhiza in cowpeas. Trans Br Mycol Soc 74:247–241

Gupta R, Kumar P (2000) Mycorrhizal plants in response to adverse environmental conditions. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer, New York, p,p67–84

Hall IR (1978) Vesicular-arbuscular mycorrhizas on two varieties of maize and one of sweetcorn. NZ J Agric Res 21:517–519

Harrison MJ (1997) The arbuscular mycorrhizal symbiosis: an underground association. Trends Plant Sci 2:54–60

Harrison MJ (1998) Development of the arbuscular mycorrhizal symbiosis. Curr Op Plant Biol 1:360–365

Harrison MJ (1999) Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 50:361–389

Hass H, Taylor TN, Remy W (1994) Fungi from the lower Devonian Rhynia Chert; mycopariasitism. Am J Bot 81:29–37

Hayman DS (1982) Influence of soils and fertility on activity and survival of vesicular-arbuscular mycorrhizal fungi. Phytopathology 72:1119–1125

Hayman DS, Mosse B (1971) Plant growth responses to vesicular arbuscular mycorrhiza. I Growth of Endogone-inoculated plants in phosphate deficient soils. New Phytol 70:19–22.

Hepper CM (1987) Gel electrophoresis for identification of AM fungi. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade. Practical applications and research priorities. Proceedings of the 7th North American Conference on Mycorrhizae. Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Fla., pp 308–310

Hetrick BAD (1984) Ecology of VA mycorrhizal fungi. In: Powell CL, Bagyaraj DJ (eds) VA mycorrhiza. CRC, Boca Raton, Fla., pp 35–55

Hetrick BAD, Kitt DG, Wilson GT (1987) Effect of drought stress on growth response in corn, sudan grass and big bluestem to *Glomus etunicatum*. New Phytol 105:403–410

Hirsch AM, Kalpulnik Y (1988) Signal transduction pathways in mycorrhizal association: Comparisons with *Rhizobium* legume symbiosis. Fung Gen Biol 23:205–212

Howeler RH, Sieverding E, Saif SR (1987) Practical aspects of mycorrhizal technology in some tropical crops and pastures. Plant Soil 100:249–283

Ibijbijen J, Urquaiga S, Ismali M, Alve JR, Boddey RM (1996) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, and nitrogen fixation of three varieties of common bean (*Phaseolus vulgaris*). New Phytol 134:353–360

Jakobsen I (1999) Transport of phosphorus and carbon in arbuscular mycorrhizas. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology, 2nd edn. Springer, Heidelberg, pp 535–542

Janos DP (1975) Effects of vesicular-arbuscular mycorrhizae on lowland tropical rain forest trees. In: Sanders FE, Mosse B, Tinker PB (eds) Endomycorrhiza. Academic, London, pp 437–446

Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002a) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jansa J, Mozafar A, Banke S, McDonald BA, Frossard E (2002b) Intra- and intersporal diversity of ITS rDNA sequences in *Glomus intraradices* assessed by cloning and sequencing, and by SSCP analysis. Mycological Research 6:670–681

Janse JM (1897) Les endophytes radicaux de quelques plantes Javanaises. Ann Jardin Bot Buitenzorg 14:53–201

Jarstfer AG, Sylvia DM (1992) Incoulum Production and inoculation technologies of vesicular arbuscular mycorrhizal fungi. In: Metting B (ed) Soil technologies: applications in agriculture, forestry and environmental management. Dekker, New York, pp 349–377

Jastrow JD (1996) Soil aggregate formation and the accrual of particulate and mineral-associated organic matter. Soil Biol Biochem 28:665–676

Johnson NC, Copeland PJ, Croolston RK, Pfleger FL (1992) Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. Agron J 84:387–390

Joner E, van Aarle IM, Vosatka M (2000) Phosphatase activity of extra-radical arbuscular mycorrhizal hyphae: a review. Plant Soil 226:199–210

Jones FR (1924) A mycorrhizal fungus in the roots of legumes and some other plants. J Agric Res 29:459–470

Kapoor R, Mukerji KG (1998) Microbial interactions in mycorrhizosphere of *Anethum graveolens* L. Phytomorphology 48:383–325

Kegler H, Gottwald J (1998) Influence of mycorrhizae on the growth and resistance of asparagus. Arch Phytopathol Plant Prot 31:435–438

Kim C, Webber DJ (1985) Distribution of VA mycorrhiza on halophytes on inland salt playas. Plant Soil 83:207–214

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148:511–517

Koide RT, Schreiner RP (1992) Regulation of the vesicular-arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 43:557–581

Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414:745–748.

Kulkarni SA, Kulkarni S, Sreenivas MN, Lulkarni S (1997) Interaction between vesicular-arbuscular (VA) mycorrhizae and *Sclerotium rolfsii* Sacc. in groundnut. Karnataka J Agric Sci 10:919–912

Lambert DH, Cole Jr H, Baker DE (1980) Variation in the responses of Alfalfa clone and cultivars to mycorrhizae and phosphorus. Crop Sci 20:615–618

Li SL, Zhao SJ, Zhao LZ, Li SL, Zhao SJ, Zhao LZ (1997) Effects of VA mycorrhizae on the growth of eggplant and cucumber and control of diseases. Acta Phytophylact Sin 24:117–120

Link HF (1809) Observations in ordines plantarum naturals. Die Gesellschaft naturforschender Freunde zu Berlin. Magazin Fur die neuesten Entdeckungen in der gesammten Naturkunde 3:33–58

Lohman ML (1927) Occurrence of mycorrhiza in lowa forest plants. University of Iowa forest plants. Univ Iowa Stud Nat Hist 11:33–58

Lu XH, Koide RT (1994) The effect of mycorrhizal infection on components of plant-growth and reproduction. New Phytol 128:211–218

McGonigle TP, Fitter AH (1990) Ecological specificity of vesicular-arbuscular mycorrhizal associations. Mycol Res 94:120–122

McGonigle TP, Miller MH (1993) Mycorrhizal development and phosphorus absorption in maize under conventional and reduced tillage. Soil Sci Soc Am J 57:1002–1006

Menge JA, Munnecke DE, Johnson ELV, Carnas DW (1978) Dosage response of the vesicular-arbuscular mycorrhizal fungi *Glomus fasciculatus* and *G. constrictus* to methyl bromide. Phytopathology 68:1368–1372

Miller RM, Jastrow JD (1990) Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biol Biochem 22:579–584

Miller RM, Jastrow JD (2000) Mycorrhizal fungi influence soil structure. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizae: molecular biology and physiology. Kluwer, Dordrecht, pp 3–18

Miller RM, Reinhardt DR, Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. Oecologia 103:17–23

Miller-Wideman MA, Watrud LS (1984) Sporulation of *Gigaspora margarita* on root cultures of tomato. Can J Microbiol 30:642–646

Morton JB, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Giagasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon 37:471–491

Morton JB, Benny GL (2001) Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with tow new genera *Archaeospora* and *Paraglomus,* based on concordant molecular and morphological characters. Mycologia 93:181–195

Mosse B (1959a) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. Trans Br Mycol Soc 42:273–286

Mosse B (1959b) Obsevations on the extramatrical mycelium of a vesicular-arbuscular endophyte. Trans Br Mycol Soc 42:439–448

Mosse B (1977) Plant growth responses to vesicular-arbuscular mycorrhiza. X. Response of stylosanthis and maize to inoculation in unsterile soils. New Phytol 78:277–288

Mosse B, Bowen GD (1968) A key to the recognition of some *Endogone* spore types. Trans Br Mycol Soc 51:469–483

Mosse B, Hepper C (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mosse B, Powell CL, Hayman DS (1976) Plant growth responses to vesicular-arbuscular mycorrhiza IX. Interactions between VA mycorrhiza rock phosphate and symbiotic nitrogen fixation. New Phytol 76:331–342

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infections in transformed root inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Nagahashi G, Douds DD, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the AM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408

Nageli C (1842) Pilze im Innern von Zellen. Linnaea 16:278–285

Nelson LL, Allen AB (1993) Restoration of *Stipa pulchra* grasslands: Effects of mycorrhizae and competition from *Avena barbata*. Restoration Ecol 2:40–50

Newman EI (1966) A method of estimating the total length of root in a sample. J Appl Ecol 3:139–145

Newsham KK, Fitter AH, Watkinson AR (1994) Root pathogenic and arbuscular mycorrhizal fungi determine fecundity of asymptomatic plants in the field. J Ecol 82:805–814

Nicolson TH (1959) Mycorrhizae in the Gramineae, I. Vesicular-arbuscular endophytes, with specical reference to the external phase. Trans Br Mycol Soc 42:421–438

Nicolson TH (1960) Mycorrhizae in the Gramineae, I. Development in different habitats, particularly sand dunes. Trans Br Mycol Soc 43:132–145

Nicolson TH (1967) Vesicular-arbuscular mycorrhiza-a universal symbiosis, Sci Prog Oxford 55:561–581

Nye P, Tinker PB (1977) Solute movement in the soil-root system. Blackwell, Oxford, p 342

Oades JM (1993) Role of biology in the formation, stabilization and degradation of soil structure. Geoderma 56:377–400

Oades JM, Waters AG (1991) Aggregate hierarchy in soils. Aust J Soil Res 29:815–828.

Olsson PA, Thingstrub I, Jakobsen I, Baath E (1999) Estimation of the biomass of arbuscular mycorrhizal fungi in a linseed field. Soil Biol Biochem 31:1879–1887

Otto G, Winkler H (1995) Colonization of rootlets of some species of Rosaceae by actinomycetes, endotrophic mycorrhiza, and endotrophic nematodes in soil conducive to specific cherry replant disease. Z Pfanzenkraft Pflanzenschutz 102:63–68

Pawlowska TE, Douds DD, Charvat I (1999) *In vitro* Propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Petri L (1903) Ricerche sul signifacto morfologica del prosporoidi (Sporangioli di Janse) nelle micorrize endotrofiche. Nuovo G Bot Ital 10:541

Peyronel B (1924) Specie di "*Endogone"* produttrici di micorize endotrofiche. Boll Stn Patol Veg Roma 5:73–75

Pfeffer CM, Bloss HE (1988) Growth and nutrition of guayule *(Parthenium argentatum)* in a saline soil as infuenced by vesicular-arbuscular mycorrhiza and phosohorus fertilization. New Phytol 108:315–321.

Pfeffer PE, Douds DD, Becard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in arbuscular mycorrhiza. Plant Physiol 120:587–598

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–160

Pirozynski KA, Dalpe Y (1989) Geological history of the Glomaceae with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–36

Pirozynski KA, Malloch DW (1975) The origin of land plants: a matter of mycotrophism. Biosystems 6:153–164

Ravnskov S, Jakobsen I (1995) Functional compatibility in arbuscular mycorrhizas measured as hyphal P transport to the plant. New Phytol 129:611–618

Ravnskov S, Larsen J, Olsson PA, Jakobsen I (1999) Effect of various organic compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. New Phytol 141:517–524

Read DJ, Koucheki HK, Hodgson J (1976) Vesicular-arbuscular mycorrhiza in natural vegetation systems. New Phytol 77:641–653

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhiza fungi within colonized roots. Mycorrhiza 10:73–80

Remy W, Taylor TN Hass H, Kerp H (1994) Four hundred million year old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843.

Rhodes LH, Gerdemann JW (1978) Translocation of calcium and phosphate by external hyphae of vesicular- arbuscular mycorrhizae. Soil Sci 126:125–126

Ricken B, Hofner W (1996) Effect of arbuscular mycorrhizal fungi (AMF) on heavy-metal tolerance of alfafa *Medicago sativa* L. and oat *Avena sativa* L. on a sewage sludge treated soil. Z Planzenernahr Bokenk 159:189–194

Rillig MC, Steinberg PD (2002) Glomalin production by an arbuscular mycorrhizal fungus: a mechanism of habitat modification? Soil Biol Biochem 34:1371–1374

Rillig MC, Wright SF, Nichols KA, Schmidt WF, Tom WF (2001) Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. Plant Soil 233:167–177.

Rose SL, Youngberg GT (1981) Tripartite associations in snowbrush (*Ceanothus velutinus)*: effect of vesicular- arbuscular mycorrhizae on growth nodulation and nitrogen fixation. Can J Bot 59:34–39

Rosendahl CN, Rosendahl S (1991) Influence of vesicular-arbuscular mycorrhizal fungi (*Glomus* spp) on the response of cucumbers *(Cucumis sativus*, L.,) to salt stress. Environ Exp Bot 31:313–318

Rosendahl S, Taylor JW (1997) Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. Mol Ecol 6:125–156

Safir GR (1968) The influence of vesicular-arbuscular mycorrhiza on the resistance of onion to Pyrenochaeta terrestris. MS Thesis, University of Illinois, Urbana

Sances FV, Ingham GR (1997) Conventional and organic alternatives to methyl bromide on California strawberries. Compost Sci Util 5:23–37

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales-application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427

Scannerini S, Bellando M (1968) Sull'ultrastuttura delle micorrhize endotrofiche di *Ornithogalum umbellatum* L. in attivita vegetative. Atti Accad Sci Torino 102:795–809

Schellenbaum L, Muller J, Boller T, Wiemken A, Schuepp H (1998) Effects of drought on non-mycorrhizal and mycorrhizal maize: changes in the pools of non-structural carbohydrates, in the activities of invertase and trehalase, and in the pools of amino acids and imino acids. New Phytol 138:59–66

Schenck NC, Graham SO, Green NE (1975) Temperature and light effect on contamination and spore germination of vesicular-arbuscular mycorrhizal fungi. Mycologia 76:1189–1192

Schlicht A (1889) Beitrag zur Kenntniss der Vebreitung und Bedetung der Mycorhizen. Landwirtsch Jahrb 18:478–560

Schreiner RP, Bethlenfalvay GJ (1995) Mycorrhizal interactions in sustainable agriculture. Crit Rev Biotechnol 15:271–287

Schreiner RP, Bethlenfalvay GJ (1997) Mycorrhizae, Biocides, and biocontrol: 3. Effects of three different fungicides on developmental stages of three AM fungi. Biol Fert Soils 24:18–26

Schuessler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res105:1413–1421

Schweiger PF, Thingstrup I, Jakobsen I (1999) Comparison of two test systems for measuring plant phosphorus uptake via arbuscular mycorrhizal fungi. Mycorrhiza 8:207–213

Shalaby AM, Hanna MM (1998) Preliminary studies on interactions between VA mycorrhizal fungus *Glomus mosseae, Bradyrhizabium japonicum* and *Pseudomonas syringae* in soybean plants. Acta Microbiol Pol 47:385–391

Sharma DD, Govindaiah Katiyar RS, Das PK, Janardhan L, Bajpai AK, Choudhry PC, Janardhan L (1995) Effect of VA mycorrhizal fungi on the incidence of major mulberrt diseases. Indian J Seric 34:34–37

Simon L, Lalonde M, Bruns T (1990) Amplification and direct sequencing of ribosomal genes form AM fungi. In: Allen MF, Williams SE (eds) Abstracts, 8th North American Conference on Mycorrhizae, University of Wyoming Agricultural Experiment Station, Laramie, Wyo., p 265

Simon L, Lalonde M, Bruns TD (1992) specific amplification of 18s fungal ribosomal genes from vesicular- arbuscular endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Simon L, Bousquet J, Roger, Levesqus C, Lalinde M (1993a) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Simon L, Levesque RC, Lalinde M (1993b) Identification of endomycorrhizal fungi colonizing roots by fluoresecent single-strand conformation polymorphism-polymerase chain reaction. Appl Environ Microbiol 59:4211–4215

Skipper HD, Smith GW (1979) Influence of soil pH on the soybean endomyceorrizha symbiosis. Plant Soil 53:559–563

Smith FA, Smith SE (1997) Structural diversity in vesicular-arbuscular mycorrhizal symbioses. New Phytol 137:373–388

Smith GS, Roncadori RW, Hussey RS (1986) Interaction of endomycorrhizal fungi, superphosphate and *Meliodogyne incognita* on cotton in microplot and field studies. J Nematol 18:208–216

Smith SE, Gianinnazzi-Pearson V (1988) Physiological interactions between symbionts in vesicular arbuscular mycorrhizal plants. Annu Rev Plant Physiol Plant Mol Biol 39:221–244

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Sparling GP, Tinker PB (1975) Mycorrhizas in pennine grassland. In: Sanders FE, Moss B, Tinker PB (eds) Endomycorrhizas. Proceedings of a symposium, University of Leeds, 22–25 July 1947, Academic, London

Sreeramula K. R, Onkarappa T, Swamy HN (1998) Biocontrol of damping off and black shank disease in tobacco nursery. Tob Res 24:1–4

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f.sp. *chrysamthemi* in an *in vitro* dual culture system with the vesicular arbuscular mycorrhizal fungus *Gloums intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

Stubblefield SP, Talyor TN, Trappe JM (1987) Fossil mycorrhizae: a case for symbiosis. Science 237:59–60

Subramanian KS, Charest C, Dwyer LM, Hamilton RI (1995) Arbuscular mycorrhizas and water relations in maize under drought stress at tasseling. New Phytol 129:643–650

Taylor TN, Remy W, Hass H, Kerp H (1995) Fossil arbuscular mycorrhizae from the early Devonian. Mycologia 87:560–573

Tester M, Smith SE, Smith FA (1987) The phemomenon of non-mycorrhizal plants. Can J Bot 65:416–431

Thaxter R (1922) A revision of the Endogoneae. Proc Am Acad Arts Sci 57:292– 348

Tinker PB (1978) Effect of vesicular-arbuscular mycorrhizae on plants nutrition and plant growth. Physiol Vegetable 16:743–775

Trappe JM (1987) Phylogenetic and ecologic aspects of mycotrophy in the angiospems from an evolutionary viewpoint. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC, Boca Raton, Fla., pp 5–25

Trappe JM, Schenck NC (1982) Taxonomy of the fungi forming endomycorrhizae. A. Vesicular-arbuscular mycorrhizal fungi (Endogonales). In: Schenck NC (ed) Methods and principles of mycorrhizal research. American Phytophological Society, St, Paul, Minn., pp 1–10

Tulasne LR, Tulasne C (1844) Fungi nonnulli hipogaei novi V. minus cogniti auct. G Bot Ital 2: 55–63

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Varma A (1995) Ecophysiology and application of arbuscular mycorrhizal fungi in arid soils. In: Varma A, Hock B (eds) Mycorrhiza:- structure, function and physiology. Springer, Heidelberg, pp 561–591

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effect of N source on pH and nutrient exchange of extrametrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza 6:247–251

Vivekanandan M, Fixen PE (1991) Cropping systems effect on mycorrhizal colonization, early growth, and phosphorus uptake of corn. Soil Sci Soc Am J 55:136–140

von Reichenbach HG, Schonbeck F (1995) Influence of VA-mycorrhiza on drought tolerance of flax *Linum usitatissimum* L. 2. Effect of VA-mycorrhiza on stomatal gas exchange, shoot water potential, phosphorus-nutrition and the accumulation of stress metabolites. J Appl Bot 69:193–188

Walker C (1987) Current concepts in the taxonomy of the Endogonaceae. Proceedings of the 7th ANCOM. IFAS, University of Florida, Gainesville, Fla

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glyco-protein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 7:97–107

Wu CG (1993) Glomales of Taiwan: III A comparative study of spore ontogeny in *Sclerocystis* (Glomaceae, Glomales). Mycotaxon 47:25–39

Wulfsohn D, Nyengaard JR (1999) Simple stereological procedure to estimate the number and dimensions of root hairs. Plant Soil 209:129–136

Xavier LJC, Germida JJ (2002) Response of lentil under controlled conditions to co-inoculation with arbuscular mycorrhizal fungi and rhizobia varying in efficacy. Soil Biol Biochem 34:181–188

Diversity, Function and Potential Applications of the Root-Associated Endophytes

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1 Diversity of Fungal Root Endophytes

Both mycorrhizal fungi and systemic fungal endophytes in the Order Clavicipitales have been extensively studied. Compared to these groups, root-associated fungal endophytes have received very little attention, even though they seem common in many ecosystems. Based on published reports, comparisons between host colonization by the root endophytes and mycorrhizal fungi from various habitats suggest that endophytes are possibly as abundant as mycorrhizas (Mandyam and Jumpponen 2005). As more reports that document the abundance of root endophytes in different habitats become available, a better understanding of the ecology and functions of these endophytes seems not only logical but critical.

The term 'endophyte' is used to describe either bacterial or fungal intracellular symbionts of plants that do not cause any visible signs of tissue damage or adverse effects on the host (Petrini 1991; Wilson 1995; Stone et al. 2000; Schulz and Boyle 2005). Fungal root endophytes are a paraphyletic group primarily occurring in the Ascomycota, although some examples also exist for Basidiomycetous endophytes (see Verma et al. 1998; Barazani et al. 2005). In this group, we usually include all root-inhabiting fungi that are considered non-mycorrhizal based on the morphology of the colonized host roots and on fungal structures produced in colonized roots typically considered indicative of dark septate endophytes (DSE). We also include fungi that produce hyaline structures when colonizing hosts intracellularly (O'Dell et al. 1993; Barrow and Aaltonen 2001; Ohki et al. 2002; Narisawa et al. 2003), but do not form typical DSE structures. These hyaline fungi can routinely be isolated from the roots of many plant species. Well-studied systemic and foliar endophytes of grasses, such as *Acremonium* sp., *Epichoë* sp. and *Neotyphodium* sp., will be excluded from this discussion.

Many of the studies of fungal root endophytes have either made no effort to identify the fungi or have focused on one fungus isolated at a single site. This gives the impression that the species diversity of fungal root endophytes is low. *Phialocephala*

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fortinii is probably the best-known fungal root endophyte (Addy et al. 2005). Much of what is known about these organisms has been extrapolated from studies conducted with *P. fortinii*. As sampling effort increases, it is becoming obvious that the diversity of fungal root endophytes may be much higher than previously thought. In this chapter, we address the resident diversity of root-associated fungi through a case study, and present data on the colonization by those fungi and on the host responses produced under laboratory conditions. We then continue with a discussion on the potential function of these endophytes beyond growth promotion, and conclude with a brief discussion on the possible applications of these endophytes.

2 The Shortgrass Steppe: A Case Study of Fungal Root Endophyte Diversity and Function

As a part of an as yet unpublished research effort that is still largely under way, we sampled five grassland and meadow sites in the Long Term Ecological Research (LTER) network in the western United States. The focus of these studies has been to gain a better understanding of the diversity of fungal root endophytes. The sampled LTER sites were Cedar Creek in Minnesota, HJ Andrews in Oregon, Jornada Range in New Mexico, Konza Prairie in Kansas, and the Shortgrass Steppe in Colorado. As a part of that research effort, the fungal cultures obtained from the roots of dominant plants at each site were divided into macromorphological groups, whose conspecificity was tested by Restriction Fragment Length Polymorphisms (RFLP) of the PCR-amplified Internal Transcribed Spacer (ITS) region of the nuclear rRNA gene repeat, and further refined by sequencing. The preliminary data analyses indicate that the communities of putative fungal endophytes were unique at each site and overlapped only marginally. We have selected one of the five field sites – Shortgrass Steppe in Colorado – for a detailed discussion, and present those findings here as a case study.

The Shortgrass Steppe is an arid grassland situated on the high plains of northeastern Colorado (1,650 m above sea level). This LTER site is dominated by *Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths and *Buchloe dactyloides* (Nutt.) Engelm. For more information on the site and its vegetation, see http://sgs.cnr.colostate.edu/. We sampled whole plants (*B. gracilis* and a dominant forb in Asteraceae, *Gutierrezia sarothrae* (Pursh) Britt. & Rusby) in order to be able to collect roots belonging to the target plants. The sampling was performed twice: early and late in the growing season in 2004. At each sampling occasion, roots from three individuals of each of the two species were washed free of soil, surface sterilized in hydrogen peroxide and plated out on low-nutrient media to isolate culturable, root-associated fungi. This culturing effort yielded a total of 54 isolates of filamentous fungi from this site. We extracted DNA from each isolate, and PCR-amplified the ITS region with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) for an ITS1-5.8S-ITS2 amplicon flanked by the small and large subunits of the rRNA gene repeat. To approximate the conspecific groupings, the ITS amplicons were digested with two endonucleases (*Alu* I and *Hind* III) and the fungal isolates were grouped based on these RFLP

phenotypes. To provide an approximate taxon affinity for the most commonly occurring RFLP phenotypes, the ITS region was also sequenced for 23 isolates using the ITS1F and ITS4 primers. The sequences were queried against GenBank using BLAST (Altschul et al. 1997) and the closest matches (Table 1) aligned in Sequencer v. 4.6 (GenCodes, Ann Arbor, Michigan). The taxon affinities were approximated using Neighbor Joining and Maximum Parsimony analyses in PAUP* 4.0 (Swofford 2002) in combination with the GenBank queries. The taxon affinities that we use here represent bootstrap supported clades (Fig. 1) and the greatest similarity to confirmed and identified accessions in GenBank.

Table 1 Approximated taxon affinities and sequence similarities of the filamentous fungi isolated from roots of *Bouteloua gracilis* and *Gutierrezia sarothrae* at the Shortgrass Steppe LTER in Colorado

Order	KSU Culture number	BLAST identification	Percent similarity	Plant host	Time of sampling
Helotiales	20345	*Cadophora luteo-olivacea* (DQ404349)	97	*G. sarothrae*	Late
	20459	*Cadophora luteo-olivacea* (DQ404349)	97	*G. sarothrae*	Late
Hypocreales	20043	*Fusarium* sp. (AY729069)	99	*B. gracilis*	Early
	20299	*Fusarium* sp. (AY729054)	99	*G. sarothrae*	Late
Pezizales	20226	*Strumella griseola* (AF485078)	87	*B. gracilis*	Early
Pleosporales	20060	*Alternaria longissima* (AF229489)	96	*B. gracilis*	Early
	20062	*Alternaria longissima* (AF229489)	97	*B. gracilis*	Early
	20346	*Alternaria longissima* (AF229489)	99	*G. sarothrae*	Late
	20414	*Alternaria longissima* (AF229489)	99	*B. gracilis*	Late
	20303	*Dreschlera* sp. (AY336133)	98	*B. gracilis*	Late
	20055	*Leptosphaeria* sp. (DQ093682)	96	*G. sarothrae*	Early
	20104	*Leptosphaeria* sp. (DQ093682)	94	*G. sarothrae*	Early
	20463	*Lophiostoma* sp. (AJ972793)	93	*G. sarothrae*	Late
	20490	*Lophiostoma* sp. (AJ972793)	93	*G. sarothrae*	Late
	20050	*Ophiosphaerella herpotricha* (U04861)	98	*G. sarothrae*	Early
	20277	*Phoma herbarum* (AY864822)	89	*B. gracilis*	Late
	20309	*Phoma herbarum* (AY864822)	89	*B. gracilis*	Late
	20328	*Phoma herbarum* (AY864822)	89	*B. gracilis*	Late
	20329	*Phoma herbarum* (AY864822)	87	*B. gracilis*	Late
Xylariales	20023	*Microdochium* sp. (AJ279477)	95	*B. gracilis*	Early
	20082	*Microdochium* sp. (AJ279477)	89	*B. gracilis*	Early
	20084	*Microdochium* sp. (AJ279477)	86	*B. gracilis*	Early
	20030	*Microdochium* sp. (AJ246155)	91	*B. gracilis*	Early

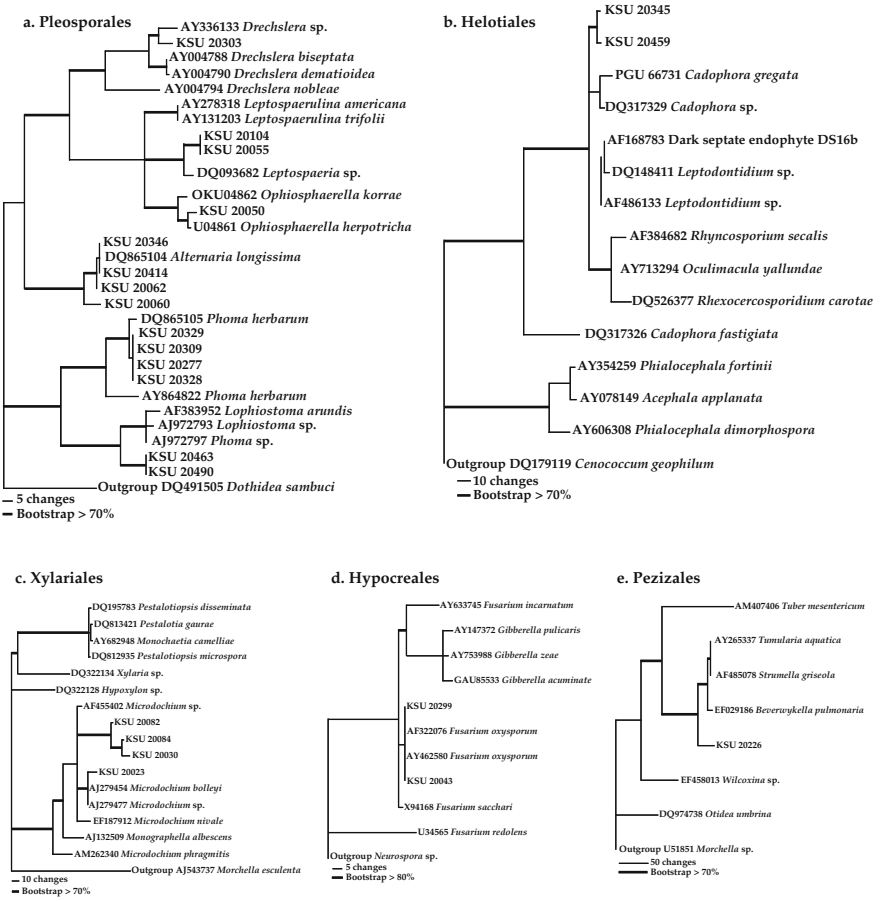

Fig. 1 Maximum parsimony bootstrap ITS trees of root endophytes isolated from the Shortgrass Steppe LTER, Colorado, USA, **a** Pleosporales, **b** Helotiales, **c** Xylariales, **d** Hypocreales, **e** Pezizales

The diversity of the root-acquired filamentous fungi was relatively high; the 54 isolates were distributed among 22 different RFLP groups, 12 of which occurred at the early season sampling and 10 at the late season sampling. The sequenced representatives of the most commonly occurring RFLP phenotypes were distributed across five orders (Helotiales, Hypocreales, Pezizales, Pleosporales, and Xylariales) and 12 genera (Fig. 1; Table 1), all within the Ascomycota. Sequence data closely correlated with the patterns observed with the RFLPs. Both datasets indicated that there were differences in the fungal communities isolated early and late in the growing season and with

regard to the plant host (Table 1). Our data suggest that roots of both *B. gracilis* and *G. sarothrae* host a different suite of fungi early and late in the growing season. We observed little overlap in RFLP groups or among sequences between the two seasons, suggesting a temporally dynamic community colonizing the roots of dominant plants at this site. Furthermore, with the exception of the most abundant RFLP groups—those with affinities to Pleosporales—most groups were limited to a single host suggesting some degree of host preference or specificity. For example, the sequenced fungal RFLP groups that represented the Pezizales or the Xylariales were exclusively obtained from *B. gracilis*, whereas the RFLP groups that represented the Helotiales or the Hypocreales were obtained from *G. sarothrae*. Only few of the isolates within the same clades (*Alternaria longissima*-like in Fig. 1a and *Fusarium*-like isolates in Fig. 1d) in our analyses were isolated from both hosts and during both sampling times.

Because of the possibility that many of the fungi isolated from plant roots may be pathogens or saprotrophs rather than true root endophytes, we screened a sub-sample of 20 isolates in a root-colonization experiment with *Allium porrum* L. (leek) in the laboratory. We grew leek plants on 1/10 strength Murashige and Skoog medium (Murashige and Skoog 1962), and inoculated 15 replicates with 20 isolates that represented the RFLP phenotypes with the highest frequencies. Each of the inoculations was compared to a paired, mock-inoculated control that received only a plug from the fungal media but no fungus. We examined roots 8 weeks after inoculation under the light microscope at 400× for the presence of intra- and intercellular hyphae and for the presence of melanized hyphae or microsclerotia. We also examined growth responses to inoculation with our isolates by measuring shoot biomass. A majority of the tested isolates failed to colonize leek roots under our experimental conditions. Furthermore, the majority of the host growth responses were either negative or neutral at the end of the eight-week incubation when compared to the paired, fungus-free control (Table 2). Inoculation with 2 of the 20 tested isolates, a *Cadophora luteo-olivacea*-like isolate and a *Phoma herbarum*-like isolate, yielded both significant and positive growth responses (Table 2) in leek when compared to the mock-inoculated controls. However, in both of these cases, only superficial or no colonization was observed. Four additional isolates, two with affinities to *Alternaria longissima*, and one to *Lophiostoma arudinis* and *Ophiosphaerella herpotricha*, produced marginally significantly ($p < 0.10$) negative effects on leek growth. Among these isolates, only the *A. longissima*-like isolate produced intracellular hyphae and microsclerotia. The remaining isolates had no visible or significant effect on host growth. Among those, the *Drechslera*-like isolate produced intracellular hyaline hyphae, a *Microdochium*-like isolate produced chlamydospores and intracellular hyphae, another *Microdochium*-like isolate produced mitospores and intracellular hyaline hyphae, and an *A. longissima*-like isolate produced microsclerotia and intracellular hyphae.

In this case study, we isolated a diverse array of fungi from roots of *B. gracilis* and *G. sarothrae*. Many of these fungi colonized the leek roots either superficially or failed to produce intra- and intercellular fungal structures indicative of typical root endophyte symbioses. Isolates that were placed in the Pleosporales with matches in GenBank and phylogenetic analyses were the most frequently observed fungi among the 54 isolates acquired from our sampling at the Shortgrass Steppe.

Table 2 Root colonization by fungi isolated from the Shortgrass Steppe LTER in Colorado and *Allium porrum* growth responses to inoculation. The growth responses were determined via comparisons among paired inoculated plants and non-inoculated controls. Non-significant host responses are considered neutral in our discussion and those that were significant according to a non-parametric median test as implemented in SAS were considered either positive or negative if inoculated hosts were larger or smaller than the controls that were mock inoculated with a inoculum from a fungus-free sterile plate with Corn Meal Agar on which the fungus was grown

BLAST identification	Isolate	Season	Colonization	Host response
Alternaria longissima (AF229489)	20060	Early	Microsclerotia, hyphae	Negative **
Alternaria longissima (AF229489)	20062	Early	Microsclerotia, hyphae	Negative**
Alternaria longissima (AF229489)	20346	Late	Microsclerotia, hyphae	Positive ^{ns}
Alternaria longissima (AF229489)	20414	Late	Superficial hyaline hyphae	Positive ^{ns}
Fusarium sp. (AY729054)	20299	Late	None	Positive ^{ns}
Cadophora luteo-olivacea (DQ404349)	20345	Late	None	Positive*
Leptosphaeria sp. (DQ093682)	20055	Early	Superficial hyphae	Negative ^{ns}
Lophiostoma sp. (AJ972793)	20463	Late	None	Negative ^{ns}
Lophiostoma sp. (AJ972793)	20490	Late	Superficial hyphae	Negative (*)
Microdochium sp. (AJ279477)	20082	Early	Spores, hyaline hyphae	Positive ^{ns}
Microdochium sp. (AJ279477)	20084	Early	Superficial spores, penetrating hyphae	Positive ^{ns}
Ophiosphaerella herpotricha (U04861)	20050	Early	None	Negative**
Phoma herbarum (AY864822)	20277	Late	None	Negative ^{ns}
Phoma herbarum (AY864822)	20309	Late	None	Positive*
Strumella griseola (AF485078)	20226	Early	None	None ^{na}

na Inoculation tests were not completed; ns $P > 0.10$; (*) $0.05 < P \leq 0.10$; * $0.01 < P \leq 0.05$; ** $P \leq 0.01$

Several of these isolates produced both melanized hyphae and microsclerotia in *A. porrum*. However, even among the isolates that produced fungal structures indicative of endophyte symbiosis, there was considerable variation. Three of the four studied *A. longissima*-like isolates were capable of colonization, whereas the fourth colonized the host only superficially. It remains uncertain whether the observed patterns indicate that true endophytes are relatively few among the root-associated fungi or that the artificial laboratory conditions preclude fungal colonization in a common host studies such as the one described here. The paucity of intracellular colonization by any particular isolate in the roots of *A. porrum* may not indicate lack of endophytic capacity in this trial, given the potential host preference that was observed among the RFLP phenotypes. Some fungal endophytes such as *P. fortinii* may be generalists and colonize a variety of hosts, whereas others—such as those examined in this case study—may exhibit some degree of host preference.

While our data may be limited in its scope and extent, they do provide some interesting background for discussion. The isolates producing fungal structures indicative of fungal root endophytes in leek roots were few in number, and those that did produce the indicative structures produced either adverse or neutral rather than positive responses as judged by the host biomass. While one must be cautious in interpreting these data, it seems that plants in their natural environment may host a greater variety of neutral and antagonistic fungi than mutualistic endophytes. Measuring biomass is only one way of examining the effect of endophytes on host plants. However, it is one of the simplest methods for screening a large number of isolates. The following section of this chapter illustrates a variety of other ways in which fungal root endophytes may affect their hosts.

Our results at the Shortgrass Steppe with *B. gracilis* and *G. sarothrae* indicate that host preference can be a factor in determining endophyte colonization. While not tested in the presented case study, the effects of the endophytes may vary among the host species. Further testing is warranted for our isolates with additional plant hosts, including native species. In addition, sampling throughout the growing season should be utilized in order to capture the full range of the root-associated fungus diversity. Finally, plant roots and soil host a diverse assemblage of organisms in natural and agricultural systems that may interact in ways that are difficult to reproduce in a laboratory setting. The effects of fungal root endophytes on plant hosts in nature may be the result of interactions with a variety other root-associated and soil-borne organisms.

3 Functions of Root Endophytes

The potential functions of root endophytes have not been as clearly defined as those of mycorrhizal fungi or clavicipitaceous grass endophytes. Our case study suggests that potential endophytes selected from a random collection of root-associated fungi are more likely to have adverse rather than positive effects on host biomass. In contrast, based on the review of limited number of published reports on the possible roles of endophytes, Mandyam and Jumpponen (2005) argue that endophyte–plant symbioses may be considered 'multifunctional.' In other words, the endophyte functions may not be limited to growth promotion or facilitation of host nutrient acquisition. For example, similarly to mycorrhizal fungi, endophytes may improve host resistance to pathogens or herbivores and enhance host stress tolerance. In the section below, we summarize briefly suggested and reported endophyte functions, and present some of our own unpublished data in support of some of the proposed potential functions.

3.1 Role of Endophytes in Host Growth and Nutrient Uptake

One and possibly the most pivotal function of mycorrhizal fungi is the facilitation of plant nutrient uptake and resultant growth stimulation. Improved nutrition and

growth may also indirectly affect the other well-known functions of mycorrhizas, such as greater stress tolerance or pathogen resistance in plants. Endophytes are also able to enhance the growth of many plant species with or without concomitant nutrient uptake (Table 3). The importance of endophyte colonization on host nutrient uptake has remained unresolved, and clear results of endophyte effects on host nutrient status are few. Inoculation of *Vulpia ciliate* ssp. *ambigua* with *Phialophora graminicola* increased P and root N levels in its roots and shoots (Newsham 1999). In an experiment with *P. fortinii* and *Pinus contorta*, Jumpponen and Trappe (1998) showed that inoculation, similarly, can enhance host nutrient acquisition from the substrate. However, even such facilitation of nutrient uptake can be variable among strains of endophytic fungi. Vohnik et al. (2003) used two strains of *P. fortinii*, neither of which had any significant effect on the shoot growth of a *Rhododendron* cultivar. However, one of the two *P. fortinii* strains increased root biomass and P levels compared to the control and to the other strain (Vohnik et al. 2005). Co-inoculation of *Rhododendron* cv. Azurro with *Oidiodendron maius* and *P. fortinii* altered N uptake and resulted in the highest foliar P concentrations (Vohnik et al. 2005).

The mechanisms of this proposed facilitation of host nutrient uptake have remained elusive. The arguments often used in support of mycorrhizal nutrient uptake may apply: extramatrical mycelium extending from the host roots may increase the surface area and therefore increase host access to soil nutrients. Barrow and Osuna (2002) present another interesting possibility. In a root exclusion experiment that controlled sources of P in the substrate, they showed that *Atriplex canescens* inoculated with *Aspergillus ustus* may have gained access to phosphate otherwise unavailable to the host plant.

Regardless of whether or not the host nutrient uptake is enhanced by the endophytes, the results from inoculation assays are variable and depend on choices of host species, endophyte taxa or strains and experimental conditions. For example, Fernando and Currah (1996) studied the effects of two DSE fungi, *Leptodontidium orchidicola* and *P. fortinii*, on host plants both under axenic resynthesis conditions and in pot cultures using monocultures of four host species or combination of these species. The results were variable depending on the growth conditions, the fungal endophyte and the host species. In the axenic resynthesis system, *L. orchidicola* damaged the host stele indicating a pathogenic interaction; in pot cultures, no such tissue damage was observed. Different strains of *L. orchidicola* also resulted in a range of growth responses from neutral to positive and negative. In the same study, *P. fortinii* did not cause any marked changes in host performance in the axenic resynthesis system. In the pot studies, however, monocultures of *Potentilla fruticosa* responded negatively to *P. fortinii*. Our unpublished studies (Mandyam and Jumpponen, unpublished) with native tallgrass prairie endophytes also suggest that growth responses are variable among the different combinations of endophyte strains and host species. *Periconia macrospinosa* is an endophyte that has been repeatedly isolated from native tallgrass prairie plants in North America (Mandyam and Jumpponen 2005). This fungal endophyte forms typical microsclerotia in the host roots. When *Andropogon gerardii*, a dominant C_4 grass, and *Elymus canadensis*, a C_3 grass, were inoculated with *P. macrospinosa* in an axenic resynthesis system,

Table 3 Effects of fungal root endophytes on plant growth and nutrition

Fungal endophyte	Host	Growth response	Nutrient uptake	Other effects	Source
Aspergillus ustus	*Atriplex canescens*	Increased root biomass, equivalent shoot biomass when plant unavailable P is provided	-	Root exclusion system used	Barrow and Osuna 2002
Cladorrhinum foecundissimum	*Gossypium hirsutum*	Increased biomass at blossom stage	Increased foliar P under P deficient condition	-	Gasoni and Gurfunkel 1997
Cryptosporiopsis sp.	*Larix decidua*	Increased root length	-	-	Schulz et al. 2002
Fusarium sp.	*Hordeum vulgare*	-	-	-	Schulz et al. 1999
L. orchidicola	*S. glauca*	Neutral in axenic system	NA	Stele damaged	Fernando and Currah 1996
Periconia macrospinosa	*Andropogon gerardii*	Increased biomass	-	-	Mandyam and Jumpponen unpublished
	Elymus canadensis	Decreased biomass	-	-	
	Brassica campestris, Raphanus sativus	Increased root growth	-	Culture filtrate used at low concentration	Shin et al. 2005
	Dryas octopetala, S. glauca, Picea glauca	Variable based on host and strain	NA	NA	
P. fortinii	*Carex* sp.	Increased biomass and levels of P in shoots	-	-	Haselwandter and Read 1982
P. fortinii	*Larix decidua*	Increased root length	-	-	Schulz et al. 2002
P. fortinii	*Pinus contorta*	+ in axenic system	Lower foliar N, P	-	Jumpponen and Trappe 1998
		Neutral in pot system	No effect	-	

(continued)

Table 3 (continued)

Fungal endophyte	Host	Growth response	Nutrient uptake	Other effects	Source
P. fortinii	*P. contorta*	Neutral with added N in pot system	Increased N	-	Jumpponen et al. 1998
		Increased with N and organic matter	-	-	
		Root biomass increased	Increased P	-	
P. fortinii	*Betula platyphylla*	Decreased growth	-	-	Hashimoto and Hyakumachi 2001
P. fortinii	*Rhododendron* sp.	Neutral	-	-	Vohnik et al. 2003
P. fortinii	*Rhododendron* sp.	Increased root biomass	Increased P	-	Vohnik et al. 2005
Phialophora graminicola	*Vulpia ciliate*	Increased short, root, total biomass; Increased root length, tillers	Increased root N Decreased shoot N Increased shoot, root and total P	-	Newsham 1999
Piriformospora indica	*Zea mays, Nicotiana tobaccum, Bacopa monniera, Artemisia annua, Petroselinum crispum, Populus tremula*	Increased growth, early rooting in tobacco calli	-	-	Varma et al. 1999
	Nicotiana tobaccum	Increased growth, seed germination and stalk elongation	No change in total N and P	-	Barazani et al. 2005
	Oryza sativa, Sorghum vulgare, Triticum sativm, Glycine max, Cicer arientinum, Solanum melongera, Dactylorhiza purpurella, D. inacrnata, D. majalis, D. fuchsia	Increased growth, greater survival rate of orchid seeds	Increased P uptake, mobilization of insoluble P	-	Singh et al. 2000 and references therein

	Spilanthes calva, Withania somnifera	Increased growth, yield, basal stem and leaf area, number of flowers and fruits, NPP	-	-	Rai et al. 2001
	Brassica oleracea, Spinancia oleracea, Brassica junacea, Arabidopsis thaliana	Increased growth, early fruiting and flowering	-	-	Kumari et al. 2003
	Adhaloda vasica	Increased biomass and root proliferation	-	-	Rai and Varma 2005
	Hordeum vulgare	Doubled biomass and increased grain yield	-	-	Waller et al. 2005
	N. tobaccum, A. thaliana	Growth increased	Total protein and N content increased in aerial parts	Increase in nitrate reductase activity	Sherameti et al. 2005
Sterile red fungus (basidiomycete)	*Triticum vulgare*	Increased shoot, root biomass and root length in non sterilized soil	-	Culture filtrate had similar effect	Sivasithamparan 1998
	Lolium rigidum	Increased shoot and root biomass in sterilized and non sterilized soils	-	-	
	Rotational crops	Increased growth	-	-	Dewan and Sivasithamparan 1989

A. gerardii growth was enhanced while *E. canadensis* growth was reduced (Fig. 2). Experimental conditions as well as the choice of hosts and/or fungal strains are clearly important drivers of the outcomes of endophyte-host interaction.

Most of the outlined examples have used fungi that form typical DSE morphologies in the roots including microsclerotia and melanized hyphae. In addition to these fungi, a number of asco- and basidiomycetes that do not form microsclerotia, but colonize host roots inter- and intracellularly, have been shown to positively affect host growth. *Cladorrhinum foecundissimum* isolated from healthy roots of *Agropyron* spp. was inoculated onto *Gossypium hirsutum* cv. Guazuncho in pot cultures (Gasoni and Gurfunkel 1997). The fungus colonized the host roots intercellularly and developed dense infection cushions in the cortex and in the root hairs. This endophyte enhanced *G. hirsutum* growth by 50% at blossom stage. Additionally, in P-deficient soils, the inoculation doubled the foliar P levels. However, similarly to many mycorrhizal experiments growth enhancement or increase in foliar P levels were not evident in high P soils.

Recently, a new basidiomycetous endophyte, *Piriformospora indica*, has gained substantial attention as a potential growth-promoting agent. This Hymenomycete colonizes the roots both inter- and intracellularly and forms coils or round bodies

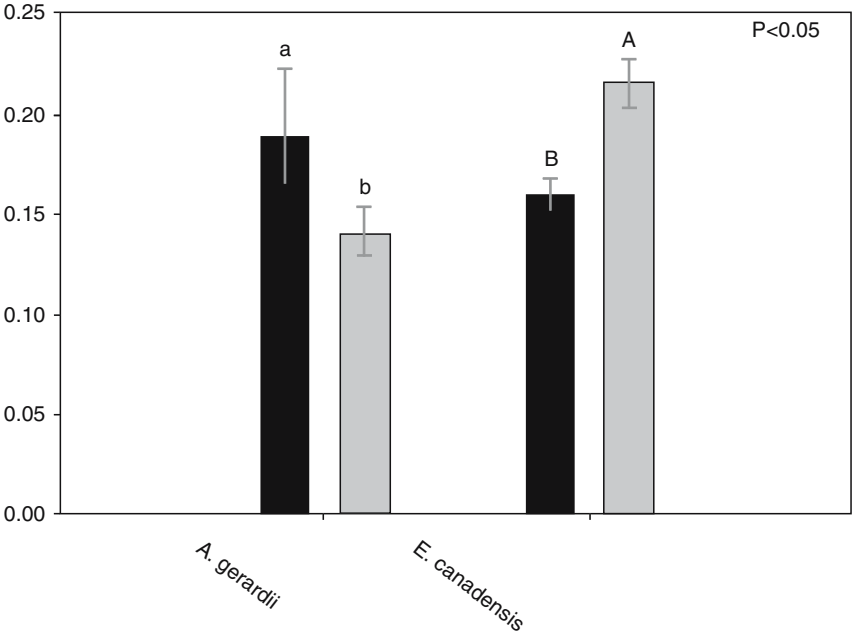

Fig. 2 Effect of *Periconia macrospinosa* on the shoot dry weight of *Andropogon gerardii* and *Elymus canadensis*. *Black bars* represent *Periconia macrospinosa* inoculated plants and *grey bars* represent control plants. Pair-wise differences (P<0.05) in *Andropogon* are indicated by *lowercase letters* and *uppercase letters* in *Elymus*, respectively. Treatments are significantly different within a species if they do not share a letter. *Bars* indicate standard error

and branches in the cortex (Verma et al. 1998; Varma et al. 1999) without any colonization of the host stele. This endophyte appears to have a broad host range. It has been shown to colonize and enhance growth of, for example, *Zea mays*, *Nicotiana tobaccum*, *Bacopa monniera*, *Artemisia annua*, *Petroselinum crispum*, *Populus tremula*, *Oryza sativa*, *Sorghum vulgare*, *Triticum sativum*, *Glycine max*, *Cicer arientinum*, *Solanum melongera*, and terrestrial orchids like *Dactylorhiza purpurella*, *D. inacrnata*, *D. majalis* and *D. fuchsia* (Singh et al. 2000; Varma et al. 1999). Barazani et al. (2005) confirmed the growth increase in *N. tobaccum* and showed that the growth promotion may be associated with improved fitness, as the inoculated plants produced more seed; similar results were also obtained in inoculation assays using *Spilanthes calva* and *Withania somnifera* (Rai et al. 2001) as well as in *Hordeum vulgare* (Waller et al. 2005).

Piriformospora may serve as a clever model system to elucidate the mechanisms of host growth and fitness promotion. A number of studies have tested its role in nutrient uptake and assimilation in symbiosis with host plants. It seems that *P. indica* is capable of mobilizing plant unavailable P by excreting extracellular phosphatases, as well as mediating uptake and translocation of labeled P via an energy dependent process (Singh et al. 2000 and references therein). It is also possible that *P. indica* is involved in N accumulation in the shoots of *N. tobaccum* and *A. thaliana* (Sherameti et al. 2005). N content in *N. tobaccum* was increased by 22%, indicating a transfer of about 60% substrate N into the plants. This N content increase was correlated with a 50% increase in nitrate reductase activity, a key enzyme in nitrate assimilation, in *N. tobaccum* and a similar 30% increase in *A. thaliana* (Sherameti et al. 2005). Whether the enhanced enzyme activity resulted in growth enhancement remains to be tested.

Endophytes may enhance growth by producing phytohormones without any apparent facilitation of host nutrient uptake or stimulation of host nutrient metabolism. The endophytic fungi may enhance biomass by producing growth hormones or inducing the host hormone production (Petrini 1991; Schulz and Boyle 2005). Simple experiments using culture extracts indicate that soluble culture extracts may stimulate host growth similarly to the actively growing fungi. The mycelial culture extract of *P. fortinii* induced a similar increase in *Larix decidua* shoot and root biomass as did the fungus itself (Römmert et al. 2002, in Schulz and Boyle 2005). Most likely the growth promotion was attributable to indole acetic acid (IAA) as the fungus synthesized the hormone *in vitro*. A similar effect has also been observed with *P. indica*. When a fungal filtrate (1% w/v) was added to maize seedlings three times a week for 4 weeks (Varma et al. 1999), shoot biomass increase was similar to that observed in inoculation experiments with living cultures of the fungus.

To summarize, many root-associated endophytes may be involved in nutrient transfer and growth enhancement in at least some cases. However, as exemplified by the case study presented above, the diversity of endophytes and their interactions with the hosts complicate generalizations, as any given combination of hosts and endophyte species or strains can behave differently. With this, we are limited to conclusions that are often presented for mycorrhizal systems (Johnson et al. 1997): the host-endophyte symbioses tend to be idiosyncratic and context dependent. In other words, the endophyte symbioses may be best judged on a case-by-case

basis without attempting overarching generalizations. As we become aware of a greater number of fungi that colonize native plants as endophytes, it appears that many common soil saprobes or benign parasites may behave like facultative endophytes.

3.2 Role of Endophytes in Resistance to Pathogens and Pests

Mycorrhizal fungi and clavicipitaceous grass endophytes can protect their hosts from pathogens and pests (Table 4). The systemic and foliar endophytes have received particular attention and can reduce herbivory by producing alkaloids toxic to insects and vertebrates (Schardl 2001). Mycorrhizal fungi are also capable of inducing resistance, and a number of mechanisms have been proposed for this resistance induction (Azcon-Aguilar and Barea 1996). Many such mechanisms of mycorrhiza-induced resistance are related to the nutritional status of the host, often correlated with mycorrhizal colonization, although some non-nutritional alternatives have also emerged (Borowicz 2001). Mycorrhizas can also mitigate the effects of herbivores, although these effects are highly variable (Gehring and Whitham 2002). To a large extent, endophytes may also be capable of improving host resistance to pathogens and pests. We will briefly review the sparse available data below and present a brief synthesis on the possible roles and mechanisms that may attribute to the altered resistance.

3.2.1 Protection from Pathogens

In the recent past, a number of reports have suggested that some endophytes can improve plant resistance to pathogens. A summary of these reports with possible mechanisms is listed in Table 4. There are at least three primary mechanisms by which endophytes can improve host resistance to pathogens (Mandyam and Jumpponen 2005).

The first mechanism is based on preemptive resource utilization by endophytes and endophyte and pathogen competition for the same resources (Lockwood 1992). This is well-illustrated in a *Fusarium oxysporum* system. A non-pathogenic *F. oxysporum* Fo47 inhibits the pathogenic *F. oxysporum* f. sp. *radicis-lycopersici* and reduces the tomato foot and root rot symptoms (Bolwerk et al. 2005). In this study, Fo47 inoculum load was 50-fold greater than that of the pathogen. The difference in inoculum loads ensured that more Fo47 spores competed with the pathogen for the same C source, thereby reducing nutrient availability to the pathogen. Both of these *Fusarium* strains exhibit similar colonization strategies. Accordingly, Fo47 can occupy and reduce the number of suitable sites for spore attachment and subsequent colonization resulting in fewer symptomatic lesions. Similar mechanisms of pathogen resistance and fewer pathogen symptoms may be applicable in other asymptomatic endophyte systems. *Phialophora radicola* var. *graminicola* may

Table 4 Role of fungal root endophytes in improving host resistance to pathogens and pests

Endophyte	Host plant	Pathogen/pest	Effects on host	Possible mechanism	Source
Piriformospora indica	*Hordeum vulgare*	*Blumeria graminis* (Powdery mildew)	Decrease of disease by 58%; hypersensitive reaction- host cell death, CW associated defense; enhanced GSH and GR activity	Systemic induction of resistance by unknown mechanism	Waller et al. 2005, 2006
	O. sativa	*Cochliobolus sativus, Fusarium culmorum*	Significant improvement of biomass in Infected plants; higher ascorbate levels in roots	Higher antioxidant levels protects from cell death	
	Spilanthes calva	*F. oxysporum, Trichophyton mentagrophytes*	Antifungal alkaloid production was enhanced	Anti microbial compounds	Rai et al. 2002
Sterile red fungus	*Triticum vulgare cv. Guntha responded the best*	*Gaeumannomyces graminis*	Lesion length and rate of lesion development reduced; results seen in field as well, root rot absent when very high inoculum densities used; culture filtrate also has similar effect	No thickening of endodermis	Sivasithamparan 1998, Kurtboke et al. 1993, Dewan and Sivasithamparan 1989
	Triticum vulgare	*Rhizoctonia solani, Pythium irregulare*	-	-	Sivasithamparan 1998
	Telopea speciosissima	*Pythophthora cryptogea*	No reduction in disease symptoms	-	
Phialophora graminicola	*Triticum vulgare*	*Gaeumannomyces graminis*	Corticular colonization by *Phialophora*, thickened endodermis; prevents pathogen entry into stele	Preemptive action, mechanical barrier; induced resistence	Deacon W 1981 in Sivasithamparam 1998, Speakman and Lewis 1978

(continued)

Table 4 (continued)

Endophyte	Host plant	Pathogen/pest	Effects on host	Possible mechanism	Source
Phialophora sp.	*Triticum vulgare*	*Gaeumannomyces graminis*	Increased seedling dry weight, increased grain yield, decreased root disease	Fast root colonization and competition for resources	Zirba et al. 1999, Mathre et al. 1998
P. fortinii	*Solanum melongena*	Verticillium dahliae	Pathogen suppression		Narisawa et al. 2002
DSE taxon LtVB3	*Brassica campestris*	*Verticillium longisporum*	External and internal symptoms reduced by 84 and 88%; CW appositions and thickenings	Indirect; DSE mycelium form mechanical barriers	Narisawa et al. 2004
Periconia macrospinosa	*A. thaliana*	*Botrytis cinerea*	Three fold reduction in disease symptoms	A systemic induced resistance like mechanism	Mandyam et al. unpublished
	No host	Bacteria	Biocidal effect	Antibacterial compounds	Kim et al. 2004, McGahren et al. 1969
Fusarium oxysporum	*Lycopersicum esculentum*	*Meliodogyne incognita*	Reduction of infection by 60%; culture filtrate toxic to adults and juveniles	Anti-microbial compounds	Hallman and Sikora 1994, 1996
Fusarium oxysporum Fo47	*Lycopersicum esculentum*	*F. oxysporum* f. sp. *radicis-lycopersici*	Pathogen colonization reduced; increased concentration of Fo47 arrested initial attachment of pathogen	Competition for same nutrients and niches; induced resistance	Bolwerk et al. 2005
Fusarium oxysporum	No host	Fungal cultures like *Phytophthora cactorum*, *Pythium ultimum*, *Rhizoctonia solani*	Radial growth reduced	Culture filtrate used at 75% concentration	Hallman and Sikora 1996
	Cucumis sativus	Virulent *F. oxysporum* f. sp. *cucumericum*	Decreased pathogen inoculum, disease suppression	Reduction of pathogen chlamydospores, competition of infection sites, induced systemic resistance	Mandeel and Baker 1991

Acremonium strictum	*Lycopersicum esculentum*	*Helicoverpa armigera*	Growth rate of larve reduced, increased developmental time, smaller pupae and suppressed moulting, reduced efficiency of food conversion	Fungal alteration of phytosterol composition	Jallow et al. 2003
Acremonium alternatum	*Brassica oleracia* var. *gemmifera*	*Plutella xylostella*	Growth rate of larvae reduced, change in female feeding preference, increased mortality, reduced efficiency of food conversion	Fungal alteration of phytosterol composition	Raps and Vidal 1998

pre-emptively reduce the colonization of *Gaeumannomyces graminis* var. *tritici* as suggested by Sivasithamparan (1998).

The second possible mechanism of pathogen control may result and stem from the chemical inhibition of root pathogens. Colonization by benign and asymptomatic endophytes may enhance the host's ability to produce biocidal compounds as in the case of *Spilanthes calva* when inoculated with *P. indica* (Rai et al. 2002). *Spilanthes calva* produces a range of antifungal compounds. Plants inoculated with *P. indica* produced extracts that were inhibitory to soil-borne pathogens (*F. oxysporum* and *Trichophyton mentagrophytes*) suggesting induction of antifungal chemical production in the host. While only scant evidence supports endophyte induction of host production of biostatics or biocides, there are many reports of endophyte culture filtrates with anti-microbial properties. A sterile red fungus, a basidiomycete, was found to produce exudates capable of lysing *G. graminis* hyphae (Sivasithamparam 1998). Pathogen exposure to the exudates reduced the size of host lesion and slowed the lesion development. *Chaetomium globosum* isolated from a barnyard grass controlled plant pathogenic fungi, including *Magnoporthe grisea* and *Puccinia recondite* (Park et al. 2005). Schulz et al. (2002), showed that, of the tested endophytes, 43% expressed antimicrobial activities while only 27% were phytopathogenic. Additionally, *Taxus cuspidate*-inhabiting *Periconia* sp., a taxon likely congeneric to the root-inhabiting *P. macrospinosa* (Mandyam and Jumpponen 2005), inhibited *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella typhimurium* with an inhibitory range that was similar to that of the commonly used antibiotic gentamycin (Kim et al. 2004). Similarly, Hallman and Sikora (1994) found that the culture filtrate of non-pathogenic *F. oxysporum* reduced the radial growth of pathogens such as *Rhizoctonia solani*, *Pythium ultimum* and *Phytophthora cactorum*. While these examples suggest that some endophytes may be capable of producing antimicrobial compounds and protect their hosts from pathogens, there is little evidence in support of this mechanism for a broader range of endophytic fungi.

The third possible mechanism in improving host resistance to pathogens by endophytes is the role of induced defense responses. This mechanism is often encountered in mycorrhizal plants where weak resistance is induced locally (Koide and Schreiner 1992) or transiently during early mycorrhizal colonization (Gianinazzi-Pearson et al. 1996). Structural modifications and induction of defense signaling can similarly result from endophyte colonization. An unidentified root-associated endophyte known as LtVB3 restricted the spread of *Verticillium longissima* in *Brassica campestris* by forming mechanical barriers, cell wall appositions and thickenings (Narisawa et al. 2004). As a result, external and internal pathogen symptoms were reduced by over 80%. Narisawa et al. (1998) also observed inhibition of *Plasmodiophora brassicae*-caused clubroot in *B. campestris* by 5% of endophytes that were isolated from the field. These endophytes included *Heteroconium chaetospira, Mortierella elongate*, *Westerdykella* sp. as well as three unknown hyaline and melanized species. Narisawa et al. (1998) proposed that superficial (*M. elongata*), cortical (hyaline and DSE fungi, *Westerdykella* sp.), or superficial and cortical (*H. chaetomium*) colonization created a mechanical barrier to the pathogens. Another example of localized and

systemic induction of host resistance is a study that used *P. indica* and barley (Waller et al. 2005). *Fusarium culmorum* KF 350 and *Cochliobolus sativus* disease severity in inoculated plants was reduced in *P. indica*-inoculated hosts. Similarly, biomass loss of the pathogen-infected plants was also drastically reduced. These positive effects correlated with higher levels of the antioxidant compound ascorbate in the roots. The antioxidants were thought to protect the cells from hypersensitive reactions. In that study, the effect of *P. indica* on a powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei*, was also studied. Similar to the effect on the root pathogens, foliar pathogenesis was reduced by over 50% and hypersensitive reactions were elicited. The authors concluded that *P. indica* inoculation induced a systemic resistance. It is likely that many tissue-penetrating endophytes may induce pathogen resistance. Our preliminary results (Mandyam et al., unpublished) indicate that *P. macrospinosa*, a common root endophyte in plants at the Konza Prairie in Kansas, USA, can reduce *Botrytis cinerea*-caused leaf spot symptoms three-fold *A. thaliana* (Fig. 3), a response most likely attributable to induced systemic resistance resulting from endophyte–host interaction.

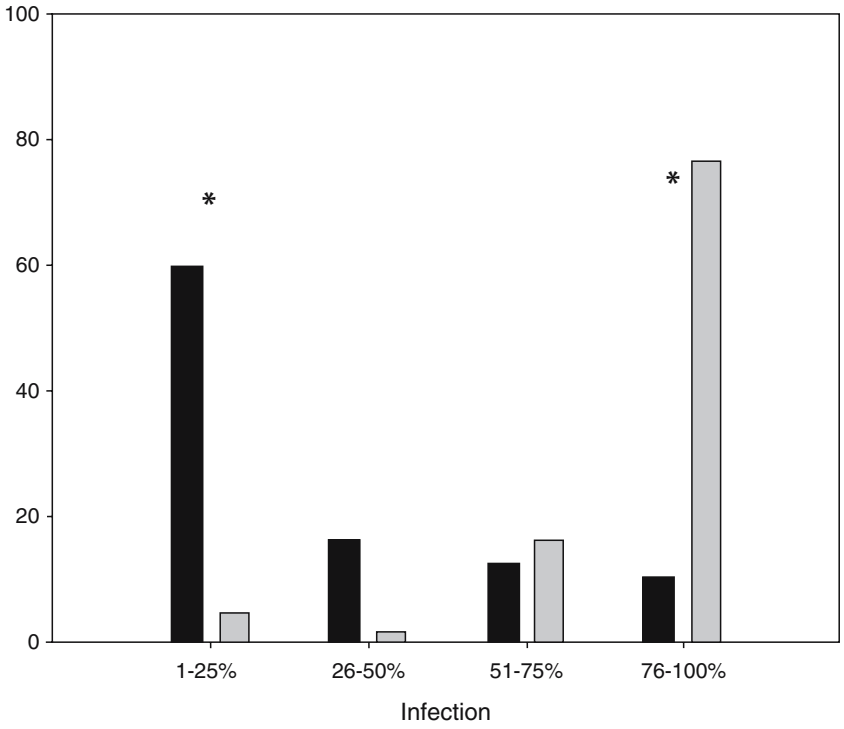

Fig. 3 Effect of *Periconia macrospinosa* on *Botrytis cinerea* necrosis in *Arabidopsis thaliana*. The *X-axis* indicates the percent leaf area necrotized by *Botrytis cinerea*. The *Y-axis* indicates the percentage of inoculated leaves. *Black bars* represent *Periconia* pre-inoculated *Arabidopsis* and *grey bars* represent control *Arabidopsis* (pre-inoculated with broth). * Indicates a three-fold difference between the treatments

In many cases, more than one of the three discussed mechanisms can act simultaneously. For example, root colonization by *P. graminicola* can pre-emptively reduce the growth of the pathogen *G. graminis* by competition for space and resources. However, it can also form mechanical barriers resulting from thickening of endodermis that inhibits colonization of the stele by the pathogen (Speakman and Lewis 1978; Deacon 1981, in Sivasithamparan 1998). Similarly, any tissue-colonizing benign organism reduces available carbon to pathogens and can occupy likely colonization sites resulting in fewer possible sites for pathogen penetration.

3.2.2 Protection from Pests and Herbivores

Mycorrhizae, and especially systemic and foliar Clavicipetalean grass endophytes, are widely known to reduce herbivory. Clavicipitaceous fungi produce toxic alkaloids against insect and vertebrate herbivores. Endophytic fungi may similarly play a role in protection of hosts from pests and herbivores. Mandyam and Jumpponen (2005), suggested three possible mechanisms by which root-associated endophytes can improve resistance of host plants to herbivores and pests. The first mechanism is based on overall improvement of plant performance by endophytes, which helps plants tolerate herbivory and sustain damage without visible effects on productivity (Gehring and Whitham 2002). Table 3 lists the instances where root endophytes can improve growth, enhance nutrient levels and improve plant fecundity whereas Table 4 lists instances where host tolerance and/or resistance to pathogens and pests has been shown to be altered.

The second possible mechanism is the alteration of plant nutritional chemistry both qualitatively and quantitatively, by altering the carbohydrate and nitrogen contents, C:N ratio and phytosterol composition (Jones and Last 1991; Bernays 1993; Schulz and Boyle 2005). The endophytes are capable of altering nutrient levels and content in host plants as discussed above. This, coupled with altered carbohydrate metabolism, can affect the host herbivore susceptibility. A few examples exist for endophytes that alter phytosterol composition in host plants and decrease herbivory. Raps and Vidal (1998) and Jallow et al. (2004) studied the interaction of non-specific endophyte with host plants to reduce insect infestation. Inoculation of *Lycopersicon esculentum* with *Acremonium strictum* reduced infestation by the tomato grub, *Helicoverpa armigera* (Jallow et al. 2004). Similarly, *Acremonium alternatum* inoculation of *Brassica olerecia* var. *gemmifera* inhibited the cabbage moth, *Plutella xylostella*, before the fungus had colonized the green foliage of the plant (Raps and Vidal 1998). In both cases, larval mortality increased and the larval growth rate was reduced among the survivors. On endophyte-inoculated *L. esculentum*, molting was also suppressed. In case of endophyte-inoculated *B. olerecia*, the moth females seemed more sensitive to the inoculation treatment, suggesting sexual differences in feeding. In both illustrated examples, the insects showed decreased efficiency in converting ingested food to biomass. While the endophytes did not appear to produce any feeding deterrents, they appeared to change the host plant phytosterol composition. Jallow et al. (2004) provided

supporting evidence and showed that *A. alternatum* can affect and alter tomato phytosterols both quantitatively and qualitatively. Raps and Vidal (1998) hypothesized that when endophytes and herbivores occupy discreet and different plant parts, the competition for nutrients will result in host-mediated differences in herbivore preferences or performance. Based on the 'sink competition hypothesis' by Larson and Whitham (1997), Raps and Vidal (1998) suggested that the greater the spatial disjunction between endophytes and herbivores, the more important will be the impact of sink build-up by endophytes on the nutritional value of food.

The third possible mechanism of host herbivore resistance is the production of feeding deterrents by the endophytes themselves. Toxic alkaloids are produced by foliar endophytes of grasses (Clay and Holah 1999; Clay 1990). Non-pathogenic *F. oxysporum*, a common root endophyte in *L. esculentum*, produces soluble toxic metabolites that are present in culture filtrates (Hallman and Sikora 1996). The filtrate has been shown to be toxic to *Meloidogyne incognita*, a root nematode. These toxic metabolites reduce nematode mobility, inactivate juveniles and are lethal within a 24-h exposure. The effects of the endophyte filtrates were reproducible in pot experiments (Hallman and Sikora 1994), indicating that the fungus also produces the metabolites *in vivo*. Mandyam and Jumpponen (2005) suggest that extensive endophyte colonization may also prevent grazing on roots. Many root-associated endophytes produce abundant melanized structures. Melanin discourages microbial grazing (Kuo and Alexander 1974; Bell and Wheeler 1986; Griffith 1994). *P. macrospinosa* extensively colonizes native grasses in the tallgrass prairie (Mandyam and Jumpponen, unpublished). As mentioned earlier, *Periconia* spp. congeneric to those from native prairies are known to produce chlorine-containing compounds that may have antibiotic properties.

4 Potential Applications of Root-Associated Endophytes

In the sections above, we have briefly reviewed the potential diversity and distribution of endophytes as well as their possible and potential functions. We continue by hypothesizing and discussing some possible applications of these root-associated fungi. Although we are unaware of any presently available commercial, agricultural and/or horticultural applications, we argue that the observed and proposed diverse functions provide a marketable base for an application development. As we outline above, some—although possibly few—endophytes are capable of the growth stimulation of many economically important species. Furthermore, the examples of induced resistance against plant pathogens and herbivores suggest potential applications as biopesticides. While use of arbuscular mycorrhizal fungi may have been limited by their obligate biotrophism, many of the endophytic fungi are readily isolated and easily maintained in pure culture facilitating their mass production. This is especially the case if conidial microfungi could be utilized for these applications. We will complete this section with precautionary notes and outline some pitfalls of these applications.

4.1 Need for Microbial Solutions in Sustainable Agriculture

In the course of the past few decades the human population has doubled. Food production has similarly increased. Use of man-made fertilizers has enabled much of the increase in the crop production. This has resulted in a 9-fold increase in N fertilization and a 4-fold increase in P fertilization (Vance 2001). Concurrent with the escalating use of commercial fertilizers, the intensity of agricultural practices has increased and a wide variety of fungicides, bactericides and pesticides are utilized in large-scale crop production. The widespread use of chemical pest-control agents can contribute to ground- and surface-water pollution.

The goals of yield-focused, large-scale agriculture remain valid, but additional priorities have emerged (Cook 1992). While industrialized countries are considering ways to reduce the environmental costs of intensive agriculture, and are seeking alternatives to traditional pesticides and fertilizers, the priorities in developing countries are somewhat different. Lack of affordable fertilizers as well as affordable biocides do not allow for intensive agriculture. Documented benefits of the arbuscular mycorrhizal symbiosis on plant performance and crop protection have fanned discussions on their use in agriculture (Menge 1983) as potential solutions for both developed and developing nations. If efficient production systems for inoculum were available, and the results were as predictable as with man-made fertilizers and biocides, sustainable microbial solutions would be more marketable. However, mycorrhizal inoculation practices are rarely compatible with industrial scale agriculture. Mycorrhizal inoculation applications are more feasible in either smaller scale agricultural and horticultural operations (Ryan and Graham 2002) or in organic farming systems (Prakash and Adholeya 2006). Further complications include the unculturability and obligate biotrophy of arbuscular mycorrhizal fungi (Wood and Cummings 1992). The production of inoculum requires growing it in symbiosis with living host plants or in cumbersome root cultures. Such inoculum production systems are a possibility, but are hindered by high costs, slow turnover and difficulty of selecting against root pathogens in long-term maintenance.

Given that adoption of inoculation practices might be of interest in both developing and developed countries, but the use of arbuscular mycorrhizal fungi is complicated by biological constraints or lack of suitable practices that would allow large scale application, we will discuss whether fungal endophytes may be a potential solution. Many of the endophytes that we discussed above are easily cultured, maintained, and manipulated.

4.2 Potential of Endophytes for Production Agriculture Applications

In the sections above, we visited the diversity of fungal root-associated endophytes. Data from our preliminary studies (Kageyama et al., unpublished) suggest that grassland ecosystems, possibly other ecosystems as well, host a diversity of known and

unknown fungi that inhabit the roots of native plants. Many of these fungi were not previously considered root-associated and/or putative endophytes. In our survey of root-associated fungi isolated at the five LTER sites, we have thus far collected a vast number of isolates distributed across over 50 taxa (Kageyama et al., unpublished). We point out that, as a result of our limited and superficial understanding of the diversity of root-inhabiting fungi, any terrestrial ecosystem has the potential of hosting taxa and/or strains that hold potential for agricultural applications. Although the numbers of the truly beneficial endophytes may be low and their discovery rare, such endophytes are likely to exist. The best available example to date is possibly *P. indica* (Varma et al. 1999; Singh et al. 2000; Shende et al. 2006). Isolated relatively recently (Verma et al. 1998), this root-associated fungus has received substantial attention as it seems to possess a broad host range, tolerates a broad range of environmental conditions, and stimulates vegetative growth as well as seed production of many economically important plants (Singh et al. 2000; Shende et al. 2006).

Plant host responses to root-inhabiting endophytes have admittedly remained somewhat unpredictable. However, our initial screenings of the larger pool of isolates from the unpublished studies, including the case study presented above, suggest that possibly up to 5–7% of the obtained root-associated fungal strains may be considered either benign or mutualistic endophytes whose mechanisms of growth stimulation remain unknown. Further studies are necessary to test if the observed colonization and the growth responses can be reproduced for agricultural plants. Given the large and unexplored diversity of these endophytes, it is likely that the natural environments host a diversity of fungi that may find an application in production systems. One should, however, exercise caution in considering using isolated fungi for horticultural and/or agricultural applications: twice as many isolates were either clearly pathogenic or antagonistic in our screening.

In addition to growth promotion, the induction of host resistance or inhibition of pathogens presents another interesting possibility for endophyte application as biopesticides, as has been proposed for some plant growth promoting rhizobacteria (Benerjee et al. 2006) and arbuscular mycorrhizal fungi (Azcon-Aguilar and Barea 1996; Maia et al. 2006). A number of mechanisms can result in the biocidal or resistance-inducing properties of root-associated micro-organisms as described above. Our preliminary experiments indicate that *P. macrospinosa* strains isolated from oak savannah and tall grass prairie often stimulate host growth simultaneously, but also induce systemic resistance to fungal pathogens (Mandyam et al., unpublished). Based on preliminary analyses of the host transcriptome, it appears that this resistance induction is mainly attributable to systemic induction of plant defense signaling pathways. Although the resistance induction is an interesting and exciting possible application, one should bear in mind that these defense reactions likely are host's responses to what it considers an attack, and result likely from penetration into the host tissue. It is important to acknowledge that these reactions may bear a carbon cost to the host. In sum, if biopesticides are to be considered, one should also maintain host growth stimulation to provide an economically viable application.

To be able to provide a viable alternative as biological fertilizer or biocide, the product should provide greater or, at the very least, a comparable yield increase or crop protection as can be obtained via conventional means when the costs of using these different approaches are accounted for. Although the endophytes may provide a variety of benefits, including increased resistance to pathogens and/or herbivores in addition to growth and yield promotion, we are not aware of a reliable cost benefit analysis that would provide a solid economical basis for selecting the growth promoting endophytes over more widely considered mycorrhizal or bacterial alternatives.

4.3 Precautionary Notes

Although the endophytes may bear a promise as biofertilizers and biopesticides, no marketable applications have emerged thus far. There are a number of complications that make product development difficult. We have previously pointed out that, while a number of records suggest arbuscular mycorrhizal benefits to many crop plants, their applications have been hindered by the difficulty of producing inocula. Selection of suitable species or strains can also be difficult: no fungal species or strain may be applicable across diverse environmental conditions and hosts. While arbuscular mycorrhizal fungi may have limited host specificity (Eom et al. 2000; Helgason et al. 2002), host specificity as well as differential growth stimulation among taxa and strains (van der Heijden et al. 1998) underlines the importance of strain and taxon selection. Because thus far only a limited number of fungi have been tested for applications under field conditions, we use *Pisolithus tinctorius*, an ectomycorrhizal basidiomycete, as an example. Strains of *P. tinctorius* selected for early conifer seedling growth promotion in southeastern United States did not perform as well as local strains and species when tested in northwestern United States (Perry et al. 1987). Similarly, strains that can be easily applied and readily colonize hosts under nursery conditions may not provide favorable effects once the seedlings are planted in the field (le Tacon et al. 1992; Jackson et al. 1995). Furthermore, it is difficult to predict how the inoculated fungi compete with the ubiquitous microbial flora present naturally in soil. If the inoculants are quickly competitively excluded, the initial growth promotion of the biofertilizer fungi may be short-lived.

Among our precautionary notes we also wish to express our concern for nation-wide and international commerce using fungal inocula. It is likely that antropogenic factors have contributed to the global spread of plant pathogens and invasive weeds. An issue that often receives little attention in considerations of biofertilizer applications is the impact that imported and possibly invasive microbes may have on the endemic communities. The inoculated fungi may persist and threaten endemic strains and species via competition (El Karkouri et al. 2006). Presently, our understanding of such dynamics is crude and no evidence exists for competitive exclusion within soil

and rhizosphere microbial communities. However, introduced aggressive and invasive strains may homogenize endemic populations and communities.

5 Conclusions

Natural ecosystems are likely to host a diversity of root-associated fungi, and some of them may be considered true endophytes. The nature of these symbioses remains unclear, although we feel confident that no single unifying generalization can be applied across the wide spectrum of these associations. Although the examples outlined indicate that some endophytes stimulate host growth and some may be involved in the facilitation of host nutrient uptake, a variety of additional host responses emerge as well. Our laboratory screening measuring shoot biomass in leeks inoculated with isolates from the Shortgrass Steppe LTER suggests that fungi that fit the definition of endophyte may be few in number and not necessarily beneficial to the host vegetative biomass accumulation. Although most studies use host growth responses as a measure of mutualism, many endophytes may also affect their hosts in other ways, such as inducing host defenses, either distally or locally, and benefit them by reducing host susceptibility to pathogens. This is natural: endophytes penetrate through the cell walls and colonize their hosts intracellularly, resulting in early host response similar to pathogen attacks. These systemic and local responses may also be analogous to those induced by mycorrhizal mutualists. However, there is still some question as to whether or not this upregulation of host defenses presents a carbon cost to the host and whether or not the induced resistance to plant pathogens allows maintenance of greater or comparable growth rates and/or greater or comparable fitness and fecundity.

The ease of culturing fungal root endophytes and their potential positive effects on hosts invites speculation on their use as biostimulants or as biopesticides. Although some endophytes, *P. indica* in particular, bear a substantial promise with their broad host ranges and documented multiple positive effects on many economically important crop species, we note that development of marketable endophyte bioproducts is difficult. At the same time, we advise caution and extensive background testing to avoid possible negative outcomes of wide applications of such endophytes. Even if such bioproduct applications are considered safe to crops and cause no adverse effects on tested plants, the responses among endemic plant and soil communities remain unknown. Widespread applications may result in homogenization of the local and endemic populations and communities of the soil- and rhizosphere-associated micro-organisms. Consequently, wide-spread applications have a potential to result in unknown losses in local and global biodiversity.

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References

Addy HD, Piercey MM, Currah RS (2005) Microfungal endophytes in roots. Can J Bot 83:1–13

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Azcon-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. Mycorrhiza 6:457–464

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Barrow JR, Aaltonen RE (2001) Evaluation of the internal colonization of *Atriplex canescens* (Prush) Nutt. roots by dark septate fungi and the influence of host physiological activity. Mycorrhiza 11:199–205

Barrow JR, Osuna P (2002) Phosphorous solubilization and uptake by dark septate fungi in four wing saltbush, *Artiplex canescens* (Prush) Nutt. J Arid Environ 51:449–451

Bernays EA (1993) Plant sterols and host-plant affiliations of herbivores. In: Bernays EA (ed) Insect-plant interactions, Vol IV. CRC, Boca Raton, pp 45–57

Bell AA, Wheeler MH (1986) Biosynthesis and functions of fungal melanins. Annu Rev Phytopathol 24:4111–451

Benerjee MN, Yesmin L, Vessey JK (2006) Plant-growth-promoting rhizobacteria as biofertilizers and biopesticides. In: Rai M (ed) Handbook of microbial biofertilizers. Food Products Press, New York, pp 137–182

Bolwerk A, Lagopodi AL, Lugtenberg BJJ, Bloemberg GV (2005) Visulaization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. Mol Plant-Microbe Interact 18:710–721

Borowicz VA (2001) Do arbuscular mycorrhizal fungi alter plant-pathogen relations? Ecology 82:3057–3068

Clay K (1990) Fungal endophytes of grasses. Annu Rev Ecol Syst 21:255–297

Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742–1744

Cook GW (1992) Fertilizing for maximum yield, 3rd. edn. MacMillan, New York

Deacon JW (1981) Ecological relationships with other fungi: competitors and hyperparasites. In: Asher MJC, Shipton PJ (eds) Biology and control of take all. Academic, London, pp 75–101

Dewan MM, Sivasithamparan K (1989) Growth promotion of rotation crop species by a sterile fungus from wheat and the effect of soil temperature and moisture on its suppression of take all. Mycol Res 93:156–160

El Karkouri K, Selosse MA, Mousain D (2006) Molecular markers detecting an ectomycorrhizal *Suillus collinitus* strain on *Pinus halepensis* roots suggest successful inoculation and persistence in Mediterranean nursery and plantation. FEMS Microbiol Ecol 55:146–158

Eom A-H, Hartnett DC, Wilson GWT (2000) Host plant species effects on arbuscular mycorrhizal communities in tallgrass prairie. Oecologia 122:435–444

Fernando AA, Currah RS (1996) A comparative study of the effects of the root endophytes *Leptodontidium orchidicola* and *Phialocephala fortinii* (Fungi Imperfecti) on the growth of some subalpine plants in culture. Can J Bot 74:1071–1078

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for higher fungi and basidiomycetes: application to identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Gasoni L, de Gurfinkel BS (1997) The endophyte *Cladorrhinum foecundissimum* in cotton roots: phosphorus uptake and host growth. Mycol Res 101:867–870

Gehring CA, Whitman TG (2002) Mycorrhizae-herbivore interactions: population and community consequences. In: van der Heijden M, Sanders I (eds) Mycorrhizal ecology. Springer, Berlin, pp 295–320

Gianinazzi-Pearson V, Dumas-Gaudot E, Golotte A, Tahiri-Alaoui A, Gianinazzi S (1996) Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. New Phytol 133:45–47

Griffith GW (1994) Phenoloxidases in *Aspergillus nidulans*: 50 years on. In: Martinelli SD, Kinghorn JR (eds) Progress in industrial microbiology, vol 29. Elsevier, Amsterdam, pp 763–788

Hallmann J, Sikora RA (1994) Influence of *Fusarium oxysporum*, a mutualistic fungal endophyte, on *Meloidogyne incognita* infection of tomato. J Plant Dis Protect 101:475–481

Hallmann J, Sikora RA (1996) Toxicity of fungal endophyte secondary metabolites to plant-parasitic nematodes and soil-borne plant-pathogenic fungi. Eur J Plant Pathol 102:155–162

Haselwandter K, Read DJ (1982) The significance of a root-fungus association in two *Carex* species of high-alpine plant communities. Oecologia 53:352–354

Hashimoto Y, Hyakumachi M (2001) Effects of isolates of ectomycorrhizal fungi and endophytic *Mycelium radicis atrovirens* that were dominant in soil from disturbed sites on growth of *Betula platyphylla* var. *japonica* seedlings. Ecol Res 15:183–191

Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW, Fitter AH (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. J Ecol 90:371–384

Jackson RM, walker C, Luff S, McEvoy C (1995) Inoculation of Sitka spuce and Douglas fir with ectomycorrhizal fungi in the United Kingdom. Mycorrhiza 5:165–173

Jallow MFA, Dugassa-Gobena D, Vidal S (2004) Indirect interaction between an unspecialized endophytic fungus and a polyphagous moth. Basic Appl Ecol 5:183–191

Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol 135:575–583

Jones CG, Last FT (1991) Ectomycorrhizae and trees: implications for aboveground herbivory. In: Barbosa B, Krischik VA, Jones CG (eds) Microbial mediation of plant-herbivore interactions. New York, Wiley-Interscience, pp 65–103

Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root colonizing fungi. New Phytol 140:295–310

Jumpponen A, Mattson KG, Trappe JM (1998) Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. Mycorrhiza 7:261–265

Kim S, Shin D, Lee T, Oh KB (2004) Periconicins, two new fusicoccane diterpenes produced by an endophytic fungus Periconia sp. with antibacterial activity. J Nat Prod 67:448–450

Koide RT, Schreiner RP (1992) Regulation of the vesicular-arbuscular mycorrhizal symbiosis. Annu Rev Plant Physiol Plant Mol Biol 43:557–581

Kumari R, Kishan H, Bhoon YK, Varma A (2003) Colonization of cruciferous plants by *Piriforma indica*. Curr Sci 85:1672–1674

Kurtboke DI, Shanker M, Rowland CY, Sivasithamparam K (1993) Responses of a sterile red fungus to soil types, wheat varities and presence of certain isolates of Streptomyces. Plant Soil 157:35–40

Kuo MJ, Alexander M (1967) Inhibition of the lysis of fungi by melanins. J Bacteriol 94:624–629

Larson KC, Whitham TG (1997) Competition between gall aphids and natural plant sinks: plant architecture affects resistance to galling. Oecologia 109:575–582

le Tacon F, Alvarex IF, Bouchard D, Henrion B, Jackson RM, Luff S, J.I. P, Pera J, Stenström E, Villeneuve N, Walker C (1992) Variations in field response of forest trees of nursery ectomycorrhizal inoculation in Europe. In: Read DJ, Lewis DH, Fitter A, Alexander IJ (eds) Mycorrhizas in ecosystems. CABI, Wallingford, pp 119–134

Lockwood JL (1992) Exploitation competition. In: Carroll GC, Wicklow DT (eds) The fungal community – its organization and role in the ecosystem. Dekker, New York, pp 243–263

Maia LC, Silveira NSS, Cavalcante UMC (2006) Interaction between arbuscular mycorrhizal fungi and root pathogens. In: Rai M (ed) Handbook of microbial biofertilizers. Food Products Press, New York, pp 325–352

Mandyam K, Jumpponen A (2005) Seeking the elusive functions of the root-colonizing dark septate endophytic fungi. Stud Mycol 53:173–18

Mandeel Q, Baker R (1991) Mechanisms involved in biocontrol of cucumber with strains of nonpathogenic *Fusarium oxysporum*. Phytopathology 81:462–69

McGahren WJ, van den Hende JH, Mitscher LA (1969) Chlorinated cyclopentenone fungitoxic metabolites from the fungus, *Sporormia affinis*. J Am Chem Soc 91:157–162

Menge JA (1983) Utilization of vesicular arbuscular mycorrhizal fungi in agriculture. New Phytol 81:553–559

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Narisawa K, Usuki F, Hashiba T (2004) Control of verticillium yellows in chinese cabbage by the dark septate endophytic fungus LtVB3. Phytopathology 94:412–418

Narisawa K, Chen M, Hashiba T, Tsuneda A (2003) Ultrastructural study on interaction between a sterile, white endophytic fungus and eggplant roots. J Gen Plant Pathol 69:292–296

Narisawa K, Kawamata H, Currah RS, Hashiba T (2002) Suppression of Verticillium wilt in eggplant by some fungal root endophytes. Eur J Plant Pathol 108:103–109

Narisawa K, Tokumasu S, Hashiba T (1998) Suppression of club root formation in Chinese cabbage by the root endophytic fungus, *Heteroconium chaetospira*. Plant Pathol 47:206–210

Newsham KK (1999) *Phialophora graminicola*, a dark septate fungus, is a beneficial associate of the grass *Vulpia ciliata* ssp. *ambigua*. New Phytol 144:517–524

O'Dell TE, Massicotte HB, Trappe Jm (1993) Root colonization of *Lupinus latifolius* Agardh., and *Pinus contorta* Dougl. by *Phialocephala fortinii* Wang and Wilcox. New Phytol 124:93–100

Ohki T, Masuya, H, Yonezawa M, Usuki F, Narisawa K, Hashiba T (2002) Colonization process of the root endophytic fungus *Heteroconium chaetospira* in roots of Chinese cabbage. Mycoscience 43:191–194

Park J-H, Choi GJ, Jang KS, Lim HK, Kim HT, Cho KY, Kim J-C (2005) Antifungal activity against plant pathogenic fungi of chaetoviridins isolated from *Chaetomium globosum*. FEMS Microbiol Lett 252:309–313

Perry DA, Molina R, Amaranthus MP (1987) Mycorrhizae, mycorhizospheres and reforestation: current knowledge and research needs. Can J For Res 17:929–940

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews J, Hirano S (eds) Microbial ecology of leaves. Springer, New York, pp, 179–191

Prakash A, Adholeya A (2006) Potential of arbuscular mycorrhizae in organic farming systems. In: Rai M (ed) Handbook of microbial biofertilizers. Food Products Press, New York, pp 223–240

Rai M, Acharya D, Singh A, et al. (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128

Rai MK, Varma A, Pandey AK (2002) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycoses 47:479–481

Rai M, Varma A (2005) Arbuscular mycorrhiz like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. J Biotechnol 52:643–650

Raps A, Vidal S (1998) Indirect effects of an unspecialized endophytic fungus on specialized plant-herbivorous insect interactions. Oecologia 114:541–547

Ryan MH, Graham JH (2002) Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil 244:263–271

Römmert AK, Oros-Sichler M, Aust H-J, Lange T, Schulz B (2002) Growth promoting effects of endophytic colonization of the roots of larch (*Larix decidua*) with *Cryptosporiopsis* sp. and *Phialophora* sp. 7th International Mycological Congress, Oslo, Norway, p 309

Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686

Schulz B, Boyle C, Draeger S, Römmert A-K, Krohn K (2002) Endophytic fungi: a source of biologically active secondary metabolites. Mycol Res 106:996–1004

Schardl CL (2001) *Epichloe festucae* and related mutualistic symbionts of grasses. Fungal Gen Biol 33:69–82

Shende S, Bhagwat K, Wadegaonkar P, Rai M, Varma A (2006) *Piriformospora indica* as a new and emerging mycofertilizer and biotizer: potentials and prospects in sustainable agriculture. In: Rai M (ed) Handbook of microbial biofertilizers. Food Products Press, New York, pp 477–496

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmuller R (2005) The endophytic fungus *Piriformospora* indica stimulates the expression of nitrate reductase and the starch degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J Bot Chem 280:26241–26247

Shin DS, Oh MN, Yang HC, Oh KB (2005) Biological characterization of periconicins, bioactive secondary metabolites, produced by *Periconia* sp OBW-115. J Microbiol Biotechnol 15:216–220

Singh A, Sharma J, Rexer KH, Varma A (2000) Plant productivity determinants beyond minerals, water and light: *Piriformospora indica* – A revolutionary plant growth promoting fungus. Curr Sci 79:1548–1554

Sivasithamparam K (1998) Root cortex – the final frontier for the biocontrol of root-rot with fungal antagonists: a case study on a sterile red fungus. Annu Rev Phytopathol 36:439–452

Speakman JB, Lewis BG (1978) Limitation of *Gaeumannomyces graminis* by wheat root responses to *Phialophora radicicola*. New Phytol 80:373–380

Stone JK, Bacon CW, White JF (2000) An overview of endophtic microbes: endophytism defined. In: Bacon CW, White JF (eds) Microbial endophytes. Dekker, New York

Swofford DL (2002) PAUP*. Phylogenetic Analysis Using Parsimony. (*and Other Methods). Version 4. Sinauer, Sunderland, Mass.

van der Heijden MGA, Boller T, Wiemken A, Sanders IA (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

Vance CP (2001) Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. Plant Physiol 127:390–397

Varma A, Verma S, Sudha, Sahay N, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol 65:2741–2744

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarbhoy A, Bisen P, Butehorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–903

Vohnik M, Lukancic S, Bahor E, Regvar M, Vosátka M, Vodnik D (2003) Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. Folia Geobot 38:191–200

Vohnik M, Albrechtova J, Vosatka M (2005) The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorous and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. Symbiosis 40:87–96

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel K-H (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DA, Sninksky JJ, White TJ (eds) PCR Protocols: a Guide to Methods and Applications. Academic Press, New York

Wilson D (1995) Endophyte-the evolution of a term, and clarification of its use and definition. Oikos 73:274–276

Wood T, Cummings B (1992) Biotechnology and the future of VAM commercialization. In: Allen MF (ed) Mycorrhizal Functioning. Chapman and Hall, London, pp 468–487

Structure, Extent and Functional Significance of Belowground Arbuscular Mycorrhizal Networks

M. Giovannetti

1 Introduction

Arbuscular mycorrhizal (AM) fungi are obligate biotrophs, which establish mutualistic symbioses with the roots of most plant species. AM fungi play a major role in soil fertility and plant nutrition, since they are able to uptake nutrients from the soil and transfer them to the host plant by means of extraradical mycelia, which explore the soil and provide an extensive surface area for water and nutrient absorption (Smith and Read 1997). Such mycelia also play a major role in interplant nutrient transfer, representing fundamental factors for the exploitation and redistribution of resources within plant communities and for the establishment, survival and maintenance of plant community diversity (Francis and Read 1984; Grime et al. 1987; Van der Heijden et al. 1998; Read 1998). AM fungi produce spores in the soil, capable of germinating in the absence of host-derived signals (Mosse 1959; Hepper and Smith 1976; Giovannetti 2000), but are unable to produce extensive mycelia without establishing a functional symbiosis with a host plant. The lack of host-regulated spore germination did not represent a selective disadvantage, since AM fungi co-evolved with their host plants for more than 400 million years (Phipps and Taylor 1996; Remy et al. 1994; Simon et al. 1993; Redecker et al. 2000), suggesting that efficient strategies must have allowed these "living fossils" to compensate for the lack of host-regulated spore germination. When no host-derived signals are present in the surrounding environment, fungal hyphae undergo a programmed growth arrest and resource reallocation, allowing long-term maintenance of viability and host-infection ability (Logi et al. 1998). The wide host range and the consequent low host specificity of AM fungi represent an efficient strategy to increase the probability of individual germlings to contact host roots. In addition to these strategies, the ability of pre-symbiotic and symbiotic mycelium to form anastomoses with self-compatible mycelia, originating wide hyphal networks may represent a fundamental mechanism for increasing the

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chances of AM symbionts to contact host roots (Giovannetti et al. 1999; Giovannetti 2001). The existence of large mycelial networks in soil means that young hyphae produced by spores germinating in the absence of the host may plug into the appropriate web as soon as the hyphal tip contacts a compatible mycelium. During the pre-symbiotic growth of AM fungi, anastomoses occur between hyphae belonging to the same individual and to different individuals of the same isolate, while hyphae of individuals belonging to different genera and species are unable to fuse, and show rejection responses, revealing AM hyphal ability to discriminate against nonself. Extraradical mycorrhizal networks produced by the same fungal isolate maintain the capacity of self recognition, evidenced by the high frequency of anastomosis between hyphae originating from the same and from different root systems (Giovannetti et al. 2001, 2004, 2006).

The aim of this chapter is to review recent developments which have contributed to the understanding of cellular and molecular events involved in self-recognition and nonself incompatibility in hyphal networks formed by AM fungi during the pre-symbiotic and symbiotic growth. Evidence for the characterization of true anastomoses and for the detection of incompatibility responses will be reviewed, together with recent results showing that the root systems of plants belonging to different species, genera and families may become interconnected by means of anastomosis formation between mycorrhizal networks, which can potentially create indefinitely large numbers of fungal linkages connecting together many plants in a community.

2 Spore Germination and Structure of Pre-symbiotic Mycelium

Spores of most *Glomus* species germinate from hyphal attachments, producing either many germ tubes, as in *G. clarum*, or a single one, as in *G. mosseae* and in *G. caledonium* (Godfrey 1957; Mosse 1959). By contrast, in *Gigaspora*, *Scutellospora* and *Acaulospora* species, germ tubes emerge directly through the spore wall (Mosse 1970; Siqueira et al. 1982). After germination, hyphae generally follow a forward, linear growth, with a strong apical dominance and regular, right-angled branches. Hyphae are thick-walled, aseptate, about 5–10 µm wide and contain many nuclei. During germination, bidirectional protoplasmic streaming of many particles – nuclei, small vacuoles, mitochondria, fat droplets, and tiny organelles – can be easily observed by using video-enhanced high-powered and two-photon fluorescence microscopy (Bago et al. 1998b; Logi et al. 1998).

Hyphae originating from germinated spores give rise to a coenocytic mycelial network whose extent is poor, although highly variable between individuals. For example, mycelial lengths in *G. caledonium* reached 30–50 mm, after 10–15 days growth on water agar, and the mean growth rate of the mycelium during the early phase was 1.97 µm/min (Logi et al. 1998), while in *G. margarita* hyphal length after 9 days growth was 18 mm and 25 mm respectively, in two different experimental conditions (Becard and Piché 1989; Gianinazzi-Pearson et al. 1989).

When growing on agar or on membranes, hyphae expanding from the primary mycelium frequently come into contact and may fuse. The use of a combination of time-lapse and video-enhanced high-powered light microscopy, image analysis, and epifluorescence microscopy has revealed that the mechanism allowing the formation of hyphal networks is represented by anastomoses between compatible hyphae. These findings evidenced that the fundamental mechanism operating in the formation of mycelial networks consists in self-recognition between compatible hyphae (Giovannetti et al. 1999).

Time-lapse microscopy allowed the complete formation of a hyphal fusion in living hyphae of AM fungi to be visualized over time, and showed that it was accomplished in 35 min after a hyphal tip showed directed growth towards another hypha. The establishment of protoplasmic continuity, the characteristic feature of true anastomoses, was evidenced by the detection of complete fusion of hyphal walls and bidirectional migration of a mass of particles between fused hyphae, which moved at the speed of 1.8 ± 0.06 µm/s through hyphal bridges established during anastomosis. Protoplasm continuity was also evidenced by the detection of succinate dehydrogenase activity, which occurred in hyphal bridges where formazan salt depositions appeared uniformly distributed along anastomosing hyphae (Fig. 1).

Moreover, no incompatibility responses, such as cytoplasm withdrawal, septa formation, vacuolization, hyphal lysis or cell wall depositions, were detected in anastomosed hyphae (Giovannetti et al. 1999). Epifluorescence microscopy revealed exchange of nuclei between hyphae of the same individual and of different individuals of the same isolate, and many nuclei were detected in the middle of fusion bridges, confirming the complete compatibility of the two mycelia. Nuclear migration occurred between hyphae belonging to the same and to different germlings of the same AM fungal isolate in three different *Glomus* species: *G. caledonium*, *G. intraradices*, *G. mosseae* (Giovannetti et al. 1999).

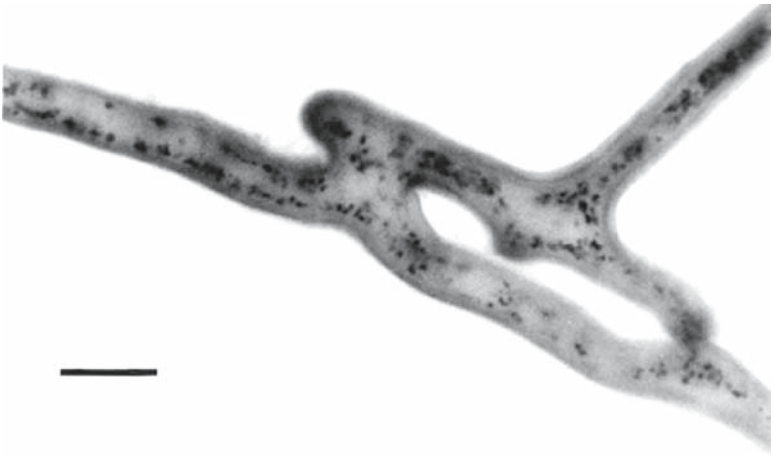

Fig. 1 Micrograph showing complete fusion of hyphal walls and the establishment of protoplasmic continuity in two anastomosing hyphae of *Glomus mosseae*, visualized by histochemical localization of SDH activity. Deposition of formazan salts are evident in hyphal bridges. *Bar* 10 µm. (Courtesy of American Society for Microbiology; Giovannetti et al. 1999)

The frequency of anastomosis between hyphae belonging to the same individuals was 57.4% and 51.4% in two different *G. mosseae* culture lines, 54.7%, 34.6% and 47.9% in three *G. caledonium* culture lines, and 58.5% and 68.7% in two *G. intraradices* culture lines. Similar results were also found in different geographical isolates of the species *G. mosseae*, with anastomosis frequency ranging from 60% in the UK isolate IMA1 to 85% in the Arizona isolate AZ225C (Giovannetti et al. 2003) (Fig. 2). Such values were obtained on hyphal contacts ranging from 91 to 242, which are relatively high numbers, given the inability of AM fungi to grow extensively in the absence of the host plant. In the experimental data, the length of pre-symbiotic mycelium varied with the different isolates, from 34.5 ± 3.5 mm in the French isolate BEG69 to 119.5 ± 14.4 mm in the UK isolate IMA1. It is interesting to note that hyphal densities detected in AM fungi, unable to grow saprophytically, ranged from 0.62 ± 0.06 to 1.3 ± 0.23 per cm of hyphal length, values comparable with those reported for saprophytic fungi, *Rhizoctonia solani* and *Gibberella fujikuroi* (Hyakumachi and Ui 1987; Correll et al. 1989; Leslie 1991).

The regular occurrence of hyphal fusions in mycelia originated from individually germinated spores suggests that the phenomenon may play an important role in maintaining the physiological continuity within each mycelium.

Anastomoses also occurred between hyphae belonging to different individuals of the same species, and their frequency was 39.6% in *G. mosseae* IMA1, 33.9% and 33.7% in two different culture lines of *G. caledonium*, and 98.1% in *G. intraradices*.

The interactions between hyphae belonging to the same germling of AM fungal species of the genera *Gigaspora* and *Scutellospora* never led to anastomoses formation. In fact, no fusions were found over 220 hyphal contacts in *G. rosea* and over 460 hyphal contacts in *S. castanea*. Other data, obtained in in vitro cultures on hyphae extending from RiT-DNA transformed carrot roots, showed the absence of anastomoses between runner hyphae of *Scutellospora reticulata*, confirming the fundamental diversity of Glomeraceae and Gigasporaceae in mycelial developmental structure (De la Providencia et al. 2005).

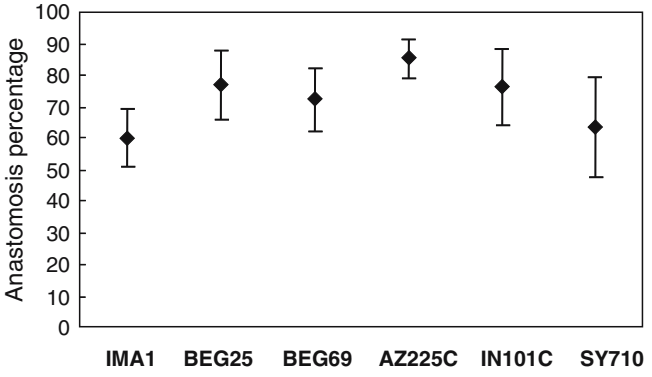

Fig. 2 Percentages of successful anastomoses with respect to total hyphal contacts in geographically different *Glomus mosseae* isolates. *Bars* represent 95% confidence limits. (Courtesy of American Society for Microbiology; Giovannetti et al. 2003)

Experimental pairings between two different species of AM fungi showed that hyphae of individuals belonging to one species never formed anastomoses with hyphae of the other, revealing hyphal ability to discriminate self from other. For example, pairings between germinated spores of *G. mosseae* and *G. caledonium* produced 90 hyphal contacts and no fusions. When hyphae of *G. mosseae* and *G. caledonium* were paired with hyphae of a species belonging to a different genus, *G. rosea*, no fusions were detected over 140 and 232 contacts, respectively. During interspecific and intergeneric hyphal interactions the responses varied from no contact interferences, to contact responses such as formation of hyphal swellings or growth of one hypha along the other without anastomosis formation, confirming that AM fungi are able to discriminate self from nonself.

Moreover, these findings demonstrate that individual mycelia originating from single germinated spores are able to recognize self entities and to fuse into them by anastomosis, generating an indeterminate network where they lose their identity and are no longer recognizable as genetically, physiologically, spatially and temporally discrete individuals. This ability to form anastomoses appears fundamental for the survival of individuals and populations of AM fungi, since it may directly affect their fitness, viability and reproductive success. In fact, young mycelia produced by spores germinating in the absence of the host might plug into other mycelia as soon as germ tubes contact compatible hyphae, originating large networks and thus enhancing their chances to contact and colonize host roots.

The ability of self-compatible hyphae to fuse and exchange nuclei has been considered of critical importance for the maintenance of genetic continuity within AM fungi, which are considered clonal organisms (Rosendhal and Taylor 1997). Since they produce single multinucleate spores, containing 1,000 to 5,000 nuclei each (Viera and Glenn 1990), and have been shown to be multigenomic (Kuhn et al. 2001), nuclear exchange during anastomoses within the same germling and between different germlings of the same isolate could represent a means for the maintenance of genetic diversity within spores, in the absence of sexual recombination (Bever and Morton 1999; Sanders 1999). Fluorescence in situ hybridization studies detected genetically different nuclei within individual spores in four species of AM fungi (Kuhn et al. 2001; Trouvelot et al. 1999). The strong genetic barriers to hyphal fusions exhibited by *G. mosseae* geographically different isolates could have the function of hindering heterokaryon formation between genetically different mycelia, thus permitting the maintenance of the fittest gene combinations. Moreover, such barriers may also prevent the exchange of cytoplasm and the spread of harmful genetic elements (Glass and Kuldau 1992; Leslie 1993).

3 Evidence for Nonself-incompatibility in Pre-symbiotic Mycelial Networks

When hyphae originating from different species or genera of AM fungi come into contact, no anastomoses are formed (Giovannetti et al. 1999; Tommerup 1988). Different intergeneric and interspecific pairings yielded 0 fusions over large numbers

of contacts, ranging from 90 in the pairing *G. mosseae-G. caledonium* to 140 in *G. mosseae-G.rosea* and 232 in *G. caledonium-G. rosea*. Interestingly, hyphal interactions produce a range of different responses, from no interference – with hyphae overgrowing each other – to the production of hyphal swellings which become empty and septate after the failure of anastomoses formation (Fig. 3). These findings, suggesting that AM fungi can recognize self entities and discriminate self from nonself, opened the way to tests of vegetative compatibility in AM fungi, already used for the identification of genetically different isolates of pathogenic, saprophytic and ectomycorrhizal fungi (Brasier 1992; Dahlberg and Stenlid 1994; Fries 1987; Leslie 1991, 1993).

Such tests, carried out on geographically different isolates of *G. mosseae*, a species of global distribution, showed that hyphal interactions between different isolates never produced anastomosis, suggesting their genetic isolation. Accordingly, hyphae intermingled without any response – compatible or incompatible – in 49–68% of contacts, while developed incompatibility reactions in 32–51% of hyphal contacts, in the different pairings (Fig. 4). Incompatibility responses were consistent with those detected in hyphae belonging to different genera and species after physical contact with hyphal swellings, localized wall thickenings, protoplasm withdrawal and retraction septa formation in the approaching hypha (Giovannetti et al. 2003), and comparable to post-fusion incompatibility reported in other fungi (Ainsworth and Rayner 1986; Leslie 1993).

The major evidence for the existence of a highly regulated system of self recognition and nonself discrimination in AM fungi was represented by the detection of

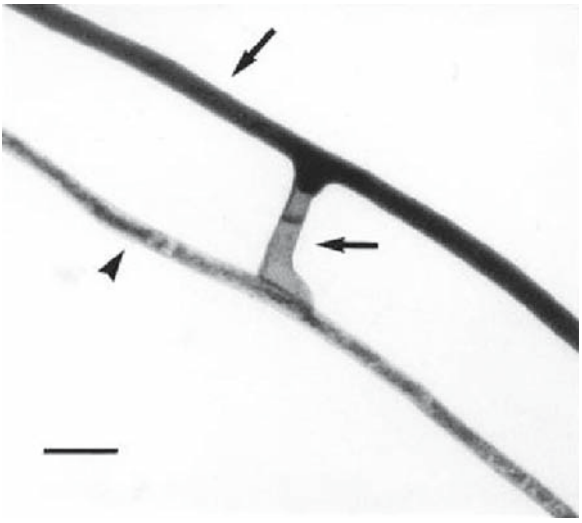

Fig. 3 Interactions between hyphae originating from two different species of arbuscular mycorrhizal fungi, *Glomus mosseae* (*upper arrow*) and *Glomus viscosum* (*arrowhead*). Note the formation of a hyphal swelling which becomes empty and septate without any anastomosis formation (*lower arrow*). *Bar* 20 µm. (Courtesy of American Society for Microbiology; Giovannetti et al. 1999)

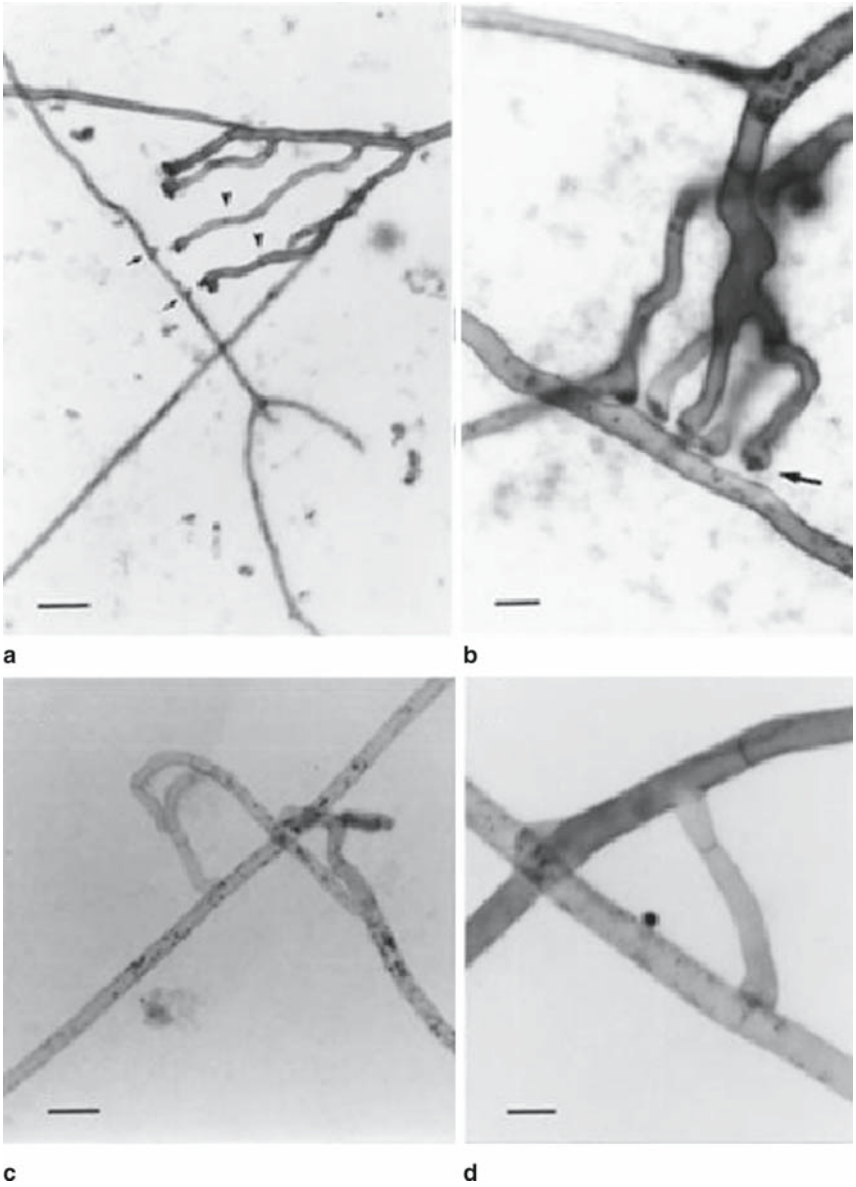

Fig. 4 Light micrographs incompatible interactions between hyphae belonging to geographically different isolates of *Glomus mosseae*, after SDH and trypan blue staining. **a** Development of multiple hyphal branches (IN101C) growing towards branch initiation sites (*arrows*) on the recipient hypha (AZ225C). Note hyphal swellings and consecutive retraction septa (*arrowheads*) produced prior to any physical contact between the hyphae. *Bar* 35µm. **b** Multiple hyphal branches (AZ225C) showing precontact protoplasm retraction and localized thickening of hyphal tips (*arrow*). *Bar* 9µm. **c** Change of growth direction in an approaching hypha (BEG25) accompanied by protoplasm withdrawal and septum formation. *Bar* 17µm. **d** Retraction septa developed by an approaching hypha (IN101C) after an incompatible interaction. *Bar* 9µm. (Courtesy of American Society for Microbiology; Giovannetti et al. 2003)

pre-contact tropism and the formation of hyphal swellings and consecutive retraction septa prior to any physical contact between neighbouring hyphae. The occurrence of hyphal tropism, previously also studied in other fungal species, *Phanerochaete velutina* and *Stereum* spp. (Ainsworth and Rayner 1986; Rayner 1991), suggests that specific recognition signals, released by interacting hyphae, are involved in interhyphal attraction and in the regulation of hyphal fusion. Nevertheless, the nature of the specific compounds acting as signals for self recognition and nonself discrimination in AM fungi remains to be unravelled.

4 Visualization of Intact Mycorrhizal Networks Spreading from Roots Colonized by AM Fungi

AM fungi living in symbiosis with host plants develop extensive extraradical hyphal networks which explore the soil, absorb mineral nutrients – N, P, Cu, Fe, K, Zn, Ca, S – and translocate them to the roots, playing a major role in plant nutrient uptake (Harrison and van Buuren 1995; Smith and Read 1997; Giovannetti and Avio 2002). Wide "mycorrhizal networks" may represent an effective source of inoculum for surrounding plants, enhancing soil infectivity and contributing to the formation and stability of soil aggregates (Tisdall and Oades 1979; Wright and Upadhyaya 1998). Moreover, due to their wide host range, AM fungi are able to colonize and interconnect plants of different species, genera and families, by means of hyphae extending from one root system to another: such extraradical fungal networks have been shown to transfer also carbon, nitrogen and phosphorus between them, representing important factors for the redistribution of resources in plant communities (Chiariello et al. 1982; Francis and Read 1984; Read 1998). Many studies, carried out after destructive extraction from the soil, have reported that the length of extraradical AM mycelium ranged from 2.6 to 54 m/g soil and from 1.6 to 1,420 cm/cm root (Tisdall and Oades 1979; Abbott and Robson 1985; Jakobsen et al. 1992; Dodd et al. 2000). Non-destructive investigations of AM extraradical mycelium, performed in root observation chambers and in vitro systems, yielded only qualitative data on its structure and growth before and after the establishment of the symbiosis (Friese and Allen 1991; Bago et al. 1998a; Jones et al. 1998). The first visualization of intact AM mycelium extending from mycorrhizal roots into the extraradical environment was obtained by means of a bidimensional model system which utilized two Millipore membranes "sandwiched" around the roots of individuals' plantlets. After only 7 days growth, a fine network of extramatrical hyphae of *G. mosseae* growing on the membranes was visible to the naked eye, and its length extended from 5,169 to 7,471 mm (hyphal length), in *Thymus vulgaris* and *Allium porrum*, respectively (Giovannetti et al. 2001).

Investigations on the mechanisms allowing the formation of extensive hyphal networks revealed that after 7 days the length of extraradical hyphae of *G. mosseae* ranged from 10 to 40 cm/cm root length, depending on the host plant.

Metabolic activity of hyphae growing in the soil, measured by using different vital stains, ranged from 63% in 6-week-old *Glomus intraradices* hyphae to 100% in 3-week-old *Glomus clarum* hyphae (Schubert et al. 1987; Hamel et al. 1990). Other data showed that the activity of extraradical mycelium ranged from 0 to 32% in hyphae extracted from soil, greatly increasing in hyphae attached to colonized roots: 96% in 6-, 9- and 13-week-old *G. mosseae* and *G. intraradices* hyphae (Sylvia 1988). Other authors reported that the length of vital extraradical hyphae ranged from 20 to 40 m m^{-1} colonized root in *Eucalyptus coccifera* seedlings inoculated with three different AM fungi (Jones et al. 1998). Our recent data are comparable with the previous values, since SDH-positive hyphae ranged from 22 to 74 m m^{-1} colonized root length in *G. mosseae*.

The experimental system devised to visualize the mycorrhizal mycelium evidenced also that the mechanism allowing the formation of the network was represented by self recognition and hyphal anastomoses. Since AM fungal hyphae showed many branches (0.86–0.97 mm^{-1}) the number of anastomoses per mm of hypha was very high (0.46–0.51), as well as their frequency, 67–77%. The frequency of anastomoses per hyphal contact was higher in extraradical mycelium (post-symbiotic) than in pre-symbiotic mycelium and also than that reported in self-anastomosing isolates of *Rhizoctonia solani* (Giovannetti et al. 1999, 2003; Hyakumuchi and Ui 1987).

It is important to stress that the viability of the mycorrhizal network was 100% and that all the anastomoses showed protoplasmic continuity and nuclear occurrence in hyphal bridges, confirming the occurrence of nuclear exchange also during fusions between extraradical (symbiotic) hyphae. Moreover, the establishment of a protoplasmic flow between fusing hyphae was shown by histochemical localization of SDH activity and by the detection of nuclei in hyphal bridges (Giovannetti et al. 2001).

Further studies should monitor the developmental structure of the mycorrhizal network and the process of formation of hyphal interconnections, since in natural situations the ability to form anastomosis by extraradical hyphae originating from different plants could represent, given the wide host range of AM fungi, the biological basis for the establishment of non-finite hyphal webs, linking together plants belonging to diverse families, genera and species (Perry et al. 1989; Read 1998).

5 Visualization of Belowground Interconnections Between Plants of Different Species, Genera and Families

AM fungi have been reported to be active in mediating nutrient transfer among plants (Chiariello et al. 1982; Francis and Read 1984; Grime et al. 1987; Watkins et al. 1996; Graves et al. 1997; Lerat et al. 2002), mainly through the extensive mycelial networks, which, due to the lack of host specificity, may link the roots of contiguous plant species (Graves et al. 1997; Read 1998; Van der Heijden et al. 1998). Recent studies showed a novel mechanism by which plants may become

interconnected, hyphal fusions between extraradical hyphae originating from different individual plant root systems of different species, genera and families (Giovannetti et al. 2004).

The bidimensional experimental system utilized allowed the visualization and quantification of fusions between contiguous mycorrhizal networks spreading from *Allium porrum* (leek) root systems and those originating from *Daucus carota* (carrot), *Gossypium hirsutum* (cotton), *Lactuca sativa* (lettuce), and *Solanum melongena* (aubergine), after inoculation with the AM symbiont *Glomus mosseae*. The use of plants belonging to different species allowed the detection of a host plant effect on the development of extraradical mycelium, since hyphal density in cotton was 6.8 mm/mm^2, value statistically different from those of all the other plant species, which ranged from 2.9 to 4.1 mm/mm^2 in lettuce and egg plant, respectively. Cotton was also the species which showed the highest interconnectedness in the mycorrhizal network: the number of anastomoses per mm^2 was 4.24 compared to values ranging from 0.86 to 1.34 for the other species.

The frequency of anastomoses between mycorrhizal networks originating from the different plant species was very high, ranging from 44% in the pairing leek-egg

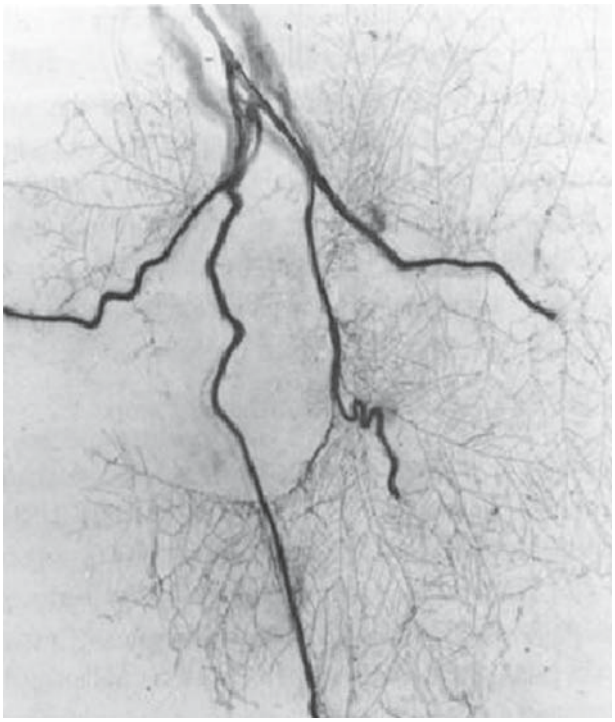

Fig. 5 Visualisation of the development of intact extraradical mycelial networks produced by *Glomus mosseae*, which grow from mycorrhizal roots of *Allium porrum* and uniformly colonize the surrounding environment. (Courtesy of New Phytologist Trust; Giovannetti et al. 2001)

plant to 49% in the pairing leek-cotton, even though lower than that between networks spreading from the same species, leek (62%).

The occurrence of true anastomoses was verified by means of SDH and DAPI staining: formazan salt depositions and nuclei were detected in the middle of hyphal bridges connecting different mycorrhizal networks, whereas no hyphal incompatibility reactions were found in interactions between hyphae connecting different mycorrhizal networks (Fig. 5).

The high rate of anastomoses formation between extraradical hyphae spreading from the root systems of different plants suggests that plant interconnectedness may be greater than previously thought, even though the high values found may be partly due to the bidimensional system, which affects the structure of the mycorrhizal network. Nevertheless, such an experimental system could be further implemented for detecting and quantifying nutrient and carbon transfer in the "web" (Fitter et al. 1998; Robinson and Fitter 1999). Moreover, due to the mechanism of anastomoses formation, and to the wide host range of AM fungi, mycorrhizal mycelium could potentially give rise to an indefinitely large network of continuous hyphae interconnecting different plants in a community and could represent a major factor in the distribution of resources in plant communities.

References

Abbott LK, Robson AD (1985) Formation of external hyphae in soil by four species of vesicular-arbuscular mycorrhizal fungi. New Phytol 99:245–255

Ainsworth AM, ADM Rayner (1986) Responses of living hyphae associated with self and non-self fusions in the basidiomycete *Phanerochaete velutina*. J Gen Microbiol 132:191–201

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Zipfel W, Williams RM, Chamberland H, Lafontaine JG, Webb WW, Piché Y (1998b) In vivo studies on the nuclear behavior of the arbuscular mycorrhizal fungus *Gigaspora rosea* grown under axenic conditions. Protoplasma 203:1–15

Becard G, Piché Y (1989) Fungal growth stimulation by CO2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bever JD, Morton J (1999) Heritable variation and mechanisms of inheritance of spore shape within a population of *Scutellospora pellucida*, an arbuscular mycorrhizal fungus. Am J Bot 86:1209–1216

Brasier C (1992) A champion thallus. Nature 356:382–383

Chiariello N, Hickman JC, Mooney HA (1982) Endomycorrhizal role for interspecific transfer of phosphorus in a community of annual plants. Science 217:941–943

Correll JC, Klittich CJR, Leslie JF (1989) Heterokaryon self-incompatibility in *Gibberella fujikuroi* (*Fusarium moniliforme*). Mycol Res 93:21–27

Dahlberg A, Stenlid J (1994) Size, distribution and biomass of genets in populations of *Suillus bovinus* (L.: Fr.) Roussel revealed by somatic incompatibility. New Phytol 128:225–234

De la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271

Dodd JC, Boddington CL, Rodriguez A, Gonzales-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

Fitter AH, Graves JD, Watkins NK, Robinson D, Scrimgeour C (1998) Carbon transfer between plants and its control in networks of arbuscular mycorrhizas. Funct Ecol 12:406–412

Francis R, Read DJ (1984) Direct transfer of carbon between plants connected by vesicular-arbuscular mycorrhizal mycelium. Nature 307:53–56

Fries N (1987) Somatic incompatibility and field distribution of the ectomycorrhizal fungus *Suillus luteus* (Boletaceae). New Phytol 107:735–739

Friese C, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphal architecture. Mycologia 83:409–418

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Giovannetti M (2000) Spore germination and pre-symbiotic mycelial growth. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 47–67

Giovannetti M (2001) Survival strategies in arbuscular mycorrhizal symbionts. In: Seckbach J (ed) Symbiosis. Kluwer, Dordrecht, pp 293–307

Giovannetti M, Avio L (2002) Biotechnology of arbuscular mycorrhizas. In: Khachatourians GG, Arora DK (eds) Applied mycology and biotechnology, vol 2: Agriculture and food production. Elsevier, Amsterdam, pp 275–310

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Fortuna P, Citernesi AS, Morini S, Nuti MP (2001) The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol 151:717–724

Giovannetti M, Sbrana C, Strani P, Agnolucci M, Rinaudo V, Avio L (2003) Genetic diversity of isolates of *Glomus mosseae* from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. Appl Environ Microbiol 69:616–624

Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal mycorrhizal networks. New Phytol 164:175–181

Giovannetti M, Avio L, Fortuna P, Pellegrino E, Sbrana C, Strani P (2006) At the root of the wood wide web self: recognition and nonself incompatibility in mycorrhizal networks. Plant Sign Behav 1:1–5

Glass NL, Kuldau GA (1992) Mating type and vegetative incompatibility in filamentous ascomycetes. Annu Rev Phytopathol 30:201–224

Godfrey RM (1957) Studies on British species of *Endogone* III. Germination of spores. Trans Br Mycol Soc 40:203–210

Graves JD, Watkins NK, Fitter AH, Robinson D, Scrimgeour C (1997) Intraspecific transfer of carbon between plants linked by a common mycorrhizal network. Plant Soil 192:153–159

Grime JP, Mackey JML, Hillier SH, Read DJ (1987) Floristic diversity in a model system using experimental microcosms. Nature 328:420–422

Hamel C, Flyes H, Smith DL (1990) Measurement of development of endomycorrhizal mycelium using three different vital stains. New Phytol 115:297–302

Harrison MJ, van Buuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:626–629

Hepper CM, Smith GA (1976) Observations on the germination of *Endogone* spores. Trans Br Mycol Soc 66:189–194

Hyakumachi M, Ui T (1987) Non-self-anastomosing isolates of *Rhizoctonia solani* obtained from fields of sugarbeet monoculture. Trans Br Mycol Soc 89:55–159

Jakobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum*. 1. Spread of hyphae and phosphorus inflow into roots. New Phytol 120:371–380

Jones MD, Durall DM, Tinker PB (1998) Comparison of arbuscular and ectomycorrhizal *Eucalyptus coccifera*: growth response, phosphorus uptake efficiency and external hyphal production. New Phytol 140:125–134

Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414:745–748

Lerat S, Gauci R, Catford JG, Vierheilig H, Piche Y, Lapointe L (2002) C-14 transfer between the spring ephemeral *Erythronium americanum* and sugar maple saplings via arbuscular mycorrhizal fungi in natural stands. Oecologia 132:181–187

Leslie JF (1991) Mating populations in *Gibberella fujikuroi* (Fusarium section Liseola). Phytopathology 81:1058–1060

Leslie JF (1993) Fungal vegetative compatibility. Annu Rev Phytopathol 31:27–150

Logi C, Sbrana C, Giovannetti M (1998) Cellular events involved in survival of individual arbuscular mycorrhizal symbionts growing in the absence of the host. Appl Environ Microbiol 64:3473–3479

Mosse B (1959) The regular germination of resting spores and some observations on the growth requirements of an *Endogone* sp. causing vesicular-arbuscular mycorrhiza. Trans Br Mycol Soc 42:273–286

Mosse B (1970) Honey-coloured, sessile *Endogone* spores. I. Life history. Arch Mikrobiol 70:167–175

Perry DA, Amaranthus MP, Borchers SI, Brainerd RE (1989) Bootstrapping in ecosystems. Bioscience 39:230–237

Phipps CJ, Taylor TN (1996) Mixed arbuscular mycorrhizae from the triassic of Antarctica. Mycologia 88:707–714

Rayner ADM (1991) The challenge of the individualistic mycelium. Mycologia 83:48–71

Read DJ (1998) The ties that bind. Nature 396:22–23

Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921

Remy W, Taylor TN, Hass H, Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Robinson D, Fitter A (1999) The magnitude and control of carbon transfer between plants linked by a common mycorrhizal network. J Exp Bot 50:9–13

Rosendhal S, Taylor JW (1997) Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP. Mol Ecol 6:821–829

Sanders I (1999) No sex please, we are fungi. Nature 399:737–739

Schubert A, Marzachì C, Mazzitelli M, Cravero MC, Bonfante-Fasolo P (1987) Development of total and viable extraradical mycelium in the vesicular-arbuscular mycorrhizal fungus *Glomus clarum* Nicol. & Schenck. New Phytol 107:183–190

Simon L, Bousquet J, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Siqueira JO, Hubbell DH, Schenck NC (1982) Spore germination and germ tube growth of a vesicular-arbuscular mycorrhizal fungus in vitro. Mycologia 74:952–959

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, San Diego

Smith FA, Jakobsen I, Smith SE (2000) Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol 147:356–366

Sylvia DM (1988) Activity of external hyphae of vesicular-arbuscular mycorrhizal fungi. Soil Biol Biochem 20:39–43

Tisdall JM, Oades JM (1979) Stabilization of soil aggregates by the root system of ryegrass. Aust J Soil Res 17:429–441

Tommerup IC (1988) The vesicular-arbuscular mycorrhizas. Adv Plant Pathol 6:81–91

Trouvelot S, van Tuinen D, Hijiri M, Gianinazzi-Pearson V (1999) Visualisation of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence in situ hybridization. Mycorrhiza 8:201–206

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Viera A, Glenn MG (1990) DNA content of vesicular-arbuscular mycorrhizal fungal spores. Mycologia 82:263–267.

Watkins NK, Fitter AH, Graves JD, Robinson D (1996) Carbon transfer between C3 and C4 plants linked by a common mycorrhizal network, quantified using stable carbon isotopes. Soil Biol Biochem 28:471–477

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198:97–107

Foraging for Resources in Arbuscular Mycorrhizal Fungi: What is an Obligate Symbiont Searching for and How is it Done?

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1 Introduction

In this review, we will present (1) a brief summary of basic concepts in foraging theory and an update on recent progress in our understanding of foraging by mycorrhizal fungi, (2) an overview of the results of some experiments testing hypotheses and models presented in a previous review on this subject (Olsson et al. 2002), and (3) an evaluation of new directions and promising approaches and methodological developments for further research on this topic. Basic processes, models and theories as they stood until year 2000 were explained in the review by Olsson et al. (2002), which also made a comparison of the foraging strategies in ectomycorrhizal and arbuscular mycorrhizal fungi.

1.1 What is Foraging?

Foraging is the most widely used term in the scientific literature when referring to the search for resources by various organisms. The theory was first developed for animals, but it has been adapted to microbes and plants although many of these organisms do not strictly look for forage. The noun forage is in most dictionaries defined as animal food from plant origin, but the verb (to forage) is used in a much wider context implying the act of looking or searching for food or provisions, or collecting diverse supplies, not necessarily defined as forage. Resources and supplies for living organisms may include (1) energy, as radiation or energy substrates, such as carbon compounds, (2) nutrients, and (3) water. The cost-benefit associated to the search for new resources has been extensively studied and modeled in animals (Stephens and Krebs 1986). Having a good strategy for the search and selection of prey, plants or nectar was recognised as an essential component of animal fitness.

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1.2 Foraging Theory

Foraging strategies are widely studied in all mobile terrestrial and aquatic macroscopic organisms, such as mammals, birds, reptiles, fishes, and large invertebrates. The concepts of optimal foraging and success as a consequence of being good at finding high quality food became highly popular in animal foraging research (MacArthur and Pianka 1966). Foraging has also been studied in sessile organisms such as plants, including both above- and belowground resources. In plants, however, there are restricted possibilities to perform a search, and most studies have used measurements of phenotypic plasticity and resource allocation to understand how plants optimize the use of their resources (Hodge 2005; Kembel et al. 2005; Yang and Midmore 2005; De Kroon and Mommer 2006). Plants can also modify their physiology, architecture and biomass distribution to make an efficient search for light, nutrients and water (Jansen et al. 2006; Walk et al. 2006; Wang et al. 2006). Clonal plants have been a popular group in foraging studies since they have the possibility of moving towards a new resource by growing in its direction, as long as the resource is available within a reasonable distance (Kelly 1990; Streitwolf-Engel et al. 1997; Cole and Mahall 2006). Some studies of plant foraging have even incorporated symbionts or interactions with soil microbes to understand how these may alter plant strategies (Cui and Caldwell 1996a; Streitwolf-Engel et al. 1997; Farley and Fitter 1999; Hodge et al.1999, 2000a, 2000b; Hodge 2001), in particular because some of them may compete with the roots for the same resources and some may provide other resources for the plant. Mycorrhizal fungi, for example, may compete with the roots for carbon, but may also provide them with phosphorus, and both actions have the potential to alter root foraging.

Foraging strategies have been studied in saprotrophic fungi, as summarized in a recommended review by Boddy (1999) and in other recent papers (Donnelly et al. 2004; Harris and Boddy 2005). Saprotrophic growth is extensive enough to allow elegant studies in which fungal foraging can be quantified through its growth pattern in heterogeneous environments (Ritz et al. 1996). Mycorrhizal fungi are more difficult to study because the symbiosis needs to be established with a host plant. Ectomycorrhizal fungi are easier to culture in this sense and have been studied more (Bending and Read 1995; Lindahl et al. 2001; Jentschke et al. 2001; Donnelly et al. 2004; Wallander et al. 2006) than arbuscular mycorrhizal fungi (AMF) (Cui and Caldwell 1996b; Olsson et al. 2002; Gavito and Olsson 2003; Nakano-Hylander and Olsson 2007).

However, culturing problems are not the only reason for the lack of studies in AMF. Most mycorrhizal research has centered on studying the capacity of AMF to transfer nutrients to the plants. The focus on nutrient exchange has distracted our attention from the fact that plants and fungi in this association are independent organisms, mainly working for their own benefit and finding resources for themselves, not for their partners (Fitter et al. 2000). Even within the plant-centered perspective, the importance of the external mycelium to explore the soil and take up mineral nutrients in AMF is obvious, but most literature is focused on nutrient

delivery to the host plant, not on the searching process. We still know very little about the capacity, plasticity, and strategies of these fungi to find their resources.

2 Resources for an Arbuscular Mycorrhizal Fungus

2.1 Carbon

Host plants are temporary sources of carbon for the fungal symbionts, and provide them with carbon, especially during growth seasons. Host plants are the only energy source for AMF (Smith and Read 1997; Nakano et al. 1999) and the roots that host them are mainly produced during vegetative growth periods. Root life span varies widely, even within a species, from days to weeks and years, depending mainly on the phenological stage. Fine roots are usually replaced and rarely last an entire growing season (Gill and Jackson 2000). Furthermore, once plants enter their reproductive stages, flowers and fruits become competing sinks for C, and C allocation belowground is usually reduced (Marschner 1995; Farrar and Jones 2000). Root production declines and many roots senesce as a consequence of the C shortage resulting from the investment in reproductive tissue (Pritchard and Rogers 2000). In addition to this, herbivory, shading, water stress induced stomatal closure, leaf shedding, etc., are potential obstacles for a continuous C flow belowground. If we add the continuous variation in C allocation belowground to root turnover and to C competition between shoot, roots and other microbes feeding from the same pool, it seems likely that plant C is an important limiting resource for AMF in most environments. AMF should, in theory, be searching continuously for alternative sources to support growth and metabolism (Olsson et al. 2002). AMF seem to be good competitors and C scavengers, as indicated by the typical reductions in root growth with increasing degree of mycorrhizal colonization (Gavito et al. 2002). Furthermore, sometimes when plants are colonized by C-demanding fungi considerable growth depressions can be seen (Eissenstat et al. 1993; Peng et al. 1993). However, once C export belowground is reduced, the fate of roots and endophytes is defined. Roots die, but AMF have the possibility of a finding a new host. If a new host is not found immediately, energy reserves are needed to persist until a host is found.

Some important new findings have strong implications for AMF C metabolism and, as a consequence, also on foraging. The complete dependency of AMF on their hosts in terms of C (Nakano et al. 1999), and evidence indicating that the extraradical mycelium relies on the export of triacylglycerides and glycogen synthesized and packed in the intraradical mycelium (Bago et al. 2000, 2003), links the foraging ability of these fungi to the continuous translocation of C compounds from the intraradical to the extraradical mycelium. The physiological differentiation of the intraradical and extraradical phases of the mycelium has strong implications for the foraging strategies of an AMF. If the extraradical phase depends on the internal

phase (Bago et al. 2002), foraging requires that the hyphal network maintains its integrity and is efficient in exporting the energy required to sustain the search and the exploitation of resources in distant parts of the mycelium. It is, therefore, extremely important to consider both host C availability and C translocation, from the intraradical to the extraradical mycelium, when studying how AMF perform the search for new resources. Pfeffer et al. (2004) have also demonstrated that the previous belief of C transfer between mycelium interconnected plants, which had already been questioned, was not correct and that the fungal symbionts did not deliver any C to other host plants. C circulates in the mycelium network in all directions and is retained and used within the mycelium.

In an experiment testing C allocation through mycelial networks from an established donor to receiver plant species, it was shown that C was not directed towards receiver seedlings to any higher degree than towards other directions (Nakano-Hylander and Olsson 2007). C was evenly distributed in the extraradical mycelium with similar labeling in extraradical mycelium as in intraradical mycelium in newly colonized seedlings. This was surprising since optimal foraging predicts that allocation of resources should increase in response to the encounter of a new resource (foraging precision). However, a test conducted with data from numerous plant species showed no support for the widespread assumption that foraging precision increases the benefit gained from growth in heterogeneous soil (Kembel et al. 2005). We are still far from having enough information to test a similar hypothesis in AMF, and clearly many more studies are needed to understand if optimal foraging, foraging precision, or foraging scale, exist in AMF and are similar to root foraging.

2.2 Water

Water is a limiting resource in dry environments and during dry seasons, and some AMF are resistant to high levels of water stress. The capacity of AMF to access water in distant and small-sized soil pores is well documented, as well as their ability to alleviate water stress in plants (Augé 2001). Water is a very little studied resource in AMF, and basically all research in mycorrhizal water relations has focused on the plants. Plants exhibit a complex set of signals and protection mechanisms to desiccation, and morphological and physiological adaptations for water search, that have not yet been explored in AMF (Ruiz-Lozano 2003; Ruiz-Lozano et al. 2006). Indirect evidence from the highly variable effects of AMF isolates on plant–water relations suggests that AMF isolates differ in their mechanisms to alleviate plant water stress (Marulanda et al. 2003). Some of these mechanisms likely involve fungal foraging and fungal plasticity, besides other complex molecular mechanisms that are currently being elucidated. Saprotrophic fungi show, for example, morphological adaptations to reduce desiccation and increase water search efficiency, such as cord formation and hyphal aggregation (Mijail and Bruhn 2005, and references therein). AMF foraging for water is becoming an increasingly relevant study area in the global climate change context.

2.3 Mineral Nutrients

The external mycelium of AMF can be stimulated by various amendments, such as organic matter (St. John et al. 1983a; Joner and Jakobsen 1995; Albertsen et al. 2006), specific organic compounds such as yeast and bovine serum albumin (Larsen and Jakobsen 1996; Ravnskov et al. 1999), or mineral nutrient-rich patches (St. John et al. 1983b). Cui and Caldwell (1996b) showed that AMF were equally efficient at acquiring P and delivering it to the roots when nutrients were located in a few enriched patches or uniformly in the soil. This implied that either hyphal proliferation or nutrient uptake capacity, or both, must have increased substantially in the nutrient-rich patches. Mycelium growth responses and nutrient uptake capacity were not measured, but the results suggest a similar plasticity to that widely reported in roots (Grime and Mackey 2002) and discussed above.

AMF foraging for mineral nutrients was studied by Gavito and Olsson (2003, 2008) in two different experimental systems, showing that mycelium responses may vary depending on how the nutrients are presented. This is in accordance to previous observations in root foraging (Grime and Mackey 2002) and to theories about AMF foraging (Tibbett 2006) that are just starting to be tested. The two foraging models will be discussed in detail in the Sect. 4 "Testing Foraging Models in AMF".

3 Foraging Activity

3.1 Growth Strategies

AMF are nonresource-unit-restricted foragers' *sensu* Boddy (1999), since they are not confined within the organic resource they are living of, and their extraradical mycelium can extend considerably beyond the host roots. The search implies risks and/or costs with no warranty for a balanced or outweighed return for the investment. Growth strategies are of fundamental importance to foraging, and studies with clonal plants can be useful to understand fungal strategies. There are two main growth strategies in plants (Schmid and Harper 1985). One is formed by plants that send out stolons on which new ramets are formed at long distances from the mother ramet. This strategy is termed guerilla type and can be found for instance in *Trifolium repens*. Other clonal plants form dense tussocks and this is called the phallanx type. It can be found for example in *Deschampsia cespitosa*. The growth strategy has critical importance for the interactions of clonal plants in communities, where the phallanx type is very stable once established in a spot, while the guerilla type has greater possibilities of spreading. The same concepts can be adopted in fungal competition, and it is clear that AMF foraging fits into the "runner" type described by Bell (1984) or the "guerrilla" pattern from Schmid and Harper (1985). Many saprotrophic fungi with dense hyphal fronts have a growth strategy more similar to the phallanx type.

No mycelium growing fronts are observed in AMF and the search is performed by long and sparse runner hyphae, with little branching. This type of foraging seems to be associated with successful foraging in heterogeneous environments with scattered resources (Donnelly and Boddy 1997). Although difficult to visualize in soil, this type of foraging is easy to observe in vitro in root organ cultures where mycelium develops in a Petri plate with nutrient rich medium. Growth *in vitro* on solid medium is likely to be different from growth in soil, but these cultures can help us to understand general strategies for how AMF might be searching in soil.

3.2 Growth and Function of AMF Extraradical Mycelium Networks

Many recent papers using monoxenic cultures convey very useful information to predict how AMF might be searching. The following is a summary of our and other researchers' observations on mycelium development *in vitro* and in soil.

Hyphae grow from roots or pieces of mycelium by sending a few runner hyphae in different directions to search the medium or the soil (Friese and Allen 1991; Bago 2000).

Runner hyphae with an adequate supply of carbon (C) from transformed roots grow several centimetres without branching until they reach the edge of the plate, with BAS (branched absorbing structures), and sometimes even spores forming directly on the runner hyphae (Fig. 1). Later, new profuse branching from the first

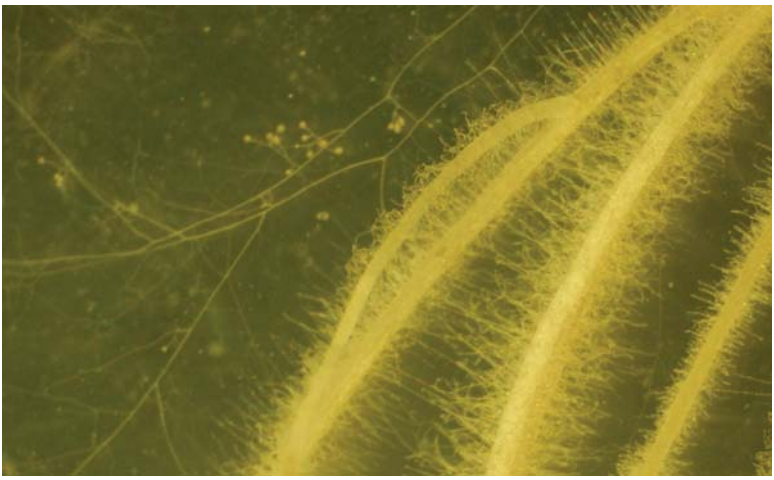


Fig. 1 Extraradical AMF mycelium networks established in monoxenic whole plant cultures between *Trifolium subterraneum* and *Glomus intraradices*. The symbiosis develops in solid Minimum medium in Petri plates. Roots are surrounded by runner hyphae connected by hyphal bridges in a young mycelium network. *Photo* Mayra E. Gavito

order group and hyphal anastomosing bridges begin to establish a loose hyphal network. Most AMF show this pattern of extraradical mycelium growth, with slight variations among the different genera in morphogenesis, architecture, anastomoses, and healing mechanisms (De Souza and Declerck 2003; De la Providencia et al. 2006; Voets et al. 2006). Some features do change when AMF grow in more heterogeneous substrates (Dodd et al. 2000), but patterns seem to be mainly the same in soil.

Extraradical hyphae with adequate carbon supply grow on average a few mm per day (Jakobsen et al. 1992; Giovanetti et al. 2000). Mycelium growing in nutrient rich medium in plates is likely foraging for carbon, not nutrients or water. When new roots are not found, the fungus keeps feeding on the medium until roots exhaust their carbon source and stop supplying carbon to the fungus. Mycelium stops growing, and when this happens BAS disappear gradually, followed by spore formation and maturation. Neutral lipids move constantly into maturing spores and, after some days, cytoplasm begins to retract from distant parts of the mycelium forming septa to close large parts of the previously active network (Giovanetti et al. 2000). In *Glomus* species, cytoplasm contracts first in distant hyphae and later in runner hyphae until most extraradical mycelium is closed (Gavito, personal observations). The cytoplasm fragmentation in the extraradical AMF mycelium when septa are formed indicates that it has only short-lasting capacity as a propagule and probably also as a foraging structure.

New evidence suggests that anastomoses and healing of injured hyphae may play an important role in the structure of hyphal networks (Voets et al. 2006) and in the foraging abilities of AMF. Mechanisms to reconnect severed sections and to maintain cytoplasm flow when mycelium is injured or cytoplasm begins to retract are essential for an efficient flux in the network, and for survival of the remaining parts of a senescing network.

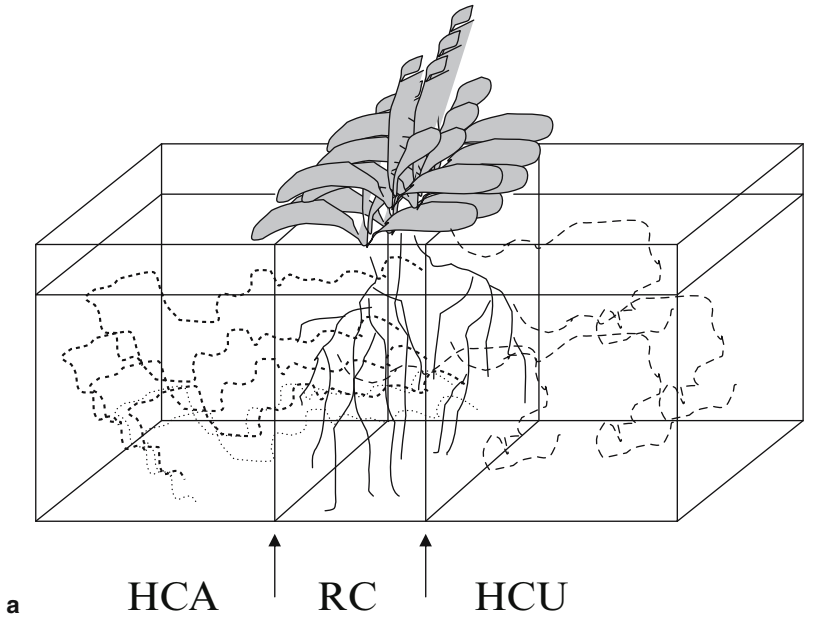
Carbon reallocation from distant parts of the mycelium to support foraging and exploit new resources found has been reported in some saprotrophic fungi (Boddy 1999). Carbon reallocation within the AMF mycelium is unknown, but some indirect evidence (Cui and Caldwell 1996b) suggests that it may exist.

4 Testing Foraging Models in AMF

Olsson et al. (2002) presented two alternative models to study foraging strategies of the AMF mycelium.

4.1 Two Dimensional Model

In the two-dimensional model, a plant colonized by AMF grows in a central compartment and two choices are presented in mycelium compartments on the right and left sides of the pot (Fig. 2a) as described in Gavito and Olsson (2003) and in the PVC cross-tube system used by Ravnskov et al. (1999) and Albertsen et al. (2006). This



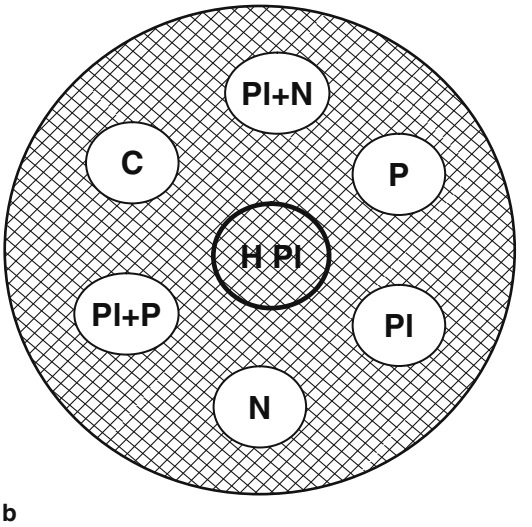


Fig. 2 Diagrams illustrating experimental models to study foraging in mycorrhizal fungi proposed by Olsson et al. (2002). **a** Two-dimensional. The host plant develops in a central compartment with one option on the right and one on the left side of the pot, so mycelium is forced to grow into the side compartments. Usually one side is amended and the other side is a control. From Gavito and Olsson (2003), printed with permission from *FEMS Microbiology Ecology*. **b** Multidimensional. The host plant grows restricted to a compartment in the middle of a large pot; mycelium develops freely and encounters the different choices after growing some distance from the plant compartment. Mycelium does not form a dense front and may choose entering or not the patches with choices. From Gavito and Olsson (2008) with permission from Applied Soil Ecology

kind of system is simple and easy to handle because it gives the possibility of sampling and changing the contents in the lateral compartments with minimum disturbance for the rest of the pot. It has the disadvantage that mycelium development is unrealistically high in the limited volume of the plant compartment and leads to the establishment of an artificial mycelium front towards the choices that are presented right in contact with dense hyphal fronts. The bidimensional model is likely to overestimate the response of the AMF. Hyphal lengths measured in these kinds of systems are higher than those observed in large pots with unrestricted root volume with the same AMF isolate used by Ravnskov and Albertsen (Gavito, personal observation), or in field measurements testing amendments (Gryndler et al. 2006).

Results from tests of this model have been useful to identify mycelium responses to inorganic and organic nutrient forms, and organic matter. They tell us little, however, about the magnitude of the response of the mycelium when it grows in a more natural manner and searches in a heterogeneous substrate with more choices available and possibilities to disregard the option presented. Gavito and Olsson (2003) used this model and found large responses to organic and inorganic nutrients provided in different combinations, in comparison with an unamended compartment located in the opposite side of the pot (Fig. 3). We found

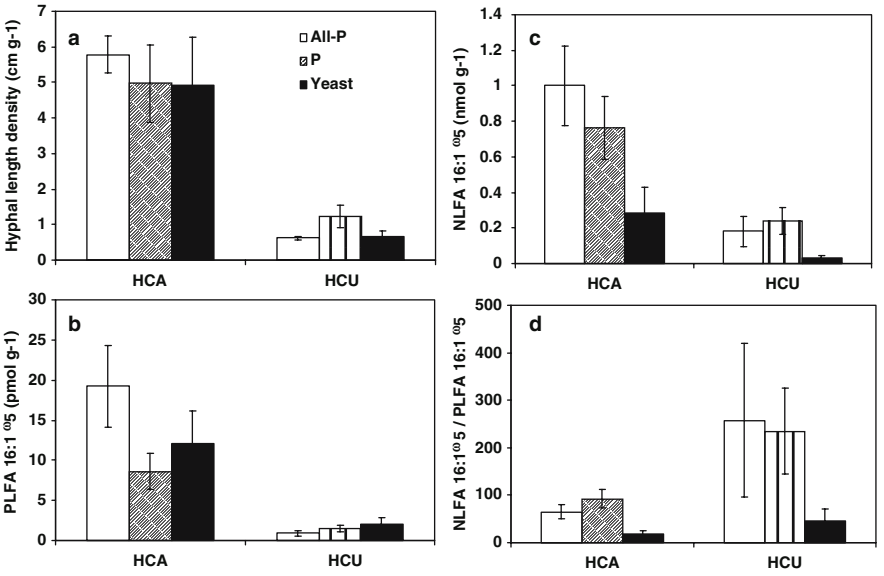


Fig. 3 Two-dimensional model. Measurements of long-term extraradical mycelium development as **a** hyphal length, **b** content of phospholipid fatty acid (PLFA) 16:1ω5, indicator for AMF biomass, **c** content of neutral lipid fatty acid (NLFA) 16:1ω5, indicator for AMF reserves. One compartment was left unamended and the other was amended with either continuous nutrient solution minus P (*All-P*), continuous only P (*P*), or a single organic amendment (*O*, as dry yeast) application. **d** PLFA/NLFA ratio as a measure of C allocation to biomass (rather "hyphal growth". Since neutral lipids is such a large part of the biomass) (PLFA), or to reserves (NLFA). From Gavito and Olsson (2003) printed with permission from *FEMS Microbiology Ecology*

that the investment made in a compartment with no nutrients was only exploratory and that most resources were sent to the amended compartment. However, the most important finding was perhaps that resources had been allocated differently within the same mycelium (Fig. 3). In the amended compartment where nutrient addition had occurred only at the start of the experiment, there had been high mycelium development while the resource was available but, when this was exhausted, and after weeks with no other supply, the mycelium began to allocate more carbon to reserves as indicated by a high neutral lipid to phospholipid ratio (Fig. 3). In the amended treatments with continuous nutrient supply, resources were used mainly for growth and exploitation of nutrients in the compartment, not for reserves. A novelty in this experiment was the use of ^{13}C to investigate short-term C allocation to AMF by measuring ^{13}C enrichment directly in the signature fatty acid 16:1ω5. The stable isotope allowed us to confirm that most C allocation prior to harvest was being used for growth in the treatments with continuous nutrient supply and for reserve lipids in the treatments with no nutrient supply or with nutrients only in a single initial dose.

4.2 Multi Dimensional Model

The second, multidimensional, model proposed by Olsson et al. (2002) implies the simultaneous exposure of the extraradical mycelium to patches of different quality. This model was tested in another study by Gavito and Olsson (2008), where a host was grown in a restricted compartment in the centre of a pot and the extraradical mycelium of *Glomus intraradices* or *Scutellospora calospora* grew radially from the central compartment to the edge of the pot. Six choices were presented at the same time in mesh bags inserted around the plant compartment (Fig. 2b). A control (no amendment), nitrogen, phosphorus, host plant, host + N, and host + P treatments were introduced and mycelium was allowed to develop for 3 weeks in the mesh bags containing the choices. The most striking result was the small response of both isolates to all choices, confirming the overestimating values obtained when using the two-dimensional model (Fig. 4).

In the two-dimensional system, however, both roots and AMF could sense the application of the amendment since there was no barrier. In the multidimensional system, there was a buffer zone to confine the choice to strictly mycelium contact. *Glomus intraradices* showed very little response to all choices, whereas *Scutellospora calospora* grew better in patches containing a host plant, especially a plant with no other amendment. All new host plants became colonized by the foraging mycelium of both isolates. However, the mycelium of *G. intraradices* was substantially weakened when the original host plant senesced and mycelium aged. *G. intraradices* colonized much less root length and formed almost no new hyphae in the patches when its mycelium was old and presumably C limited. Even in a nutrient poor substrate, the isolates did not proliferate in the N or P patches, indicating that these nutrients were not limiting and that creating a poor environment for the fungi is

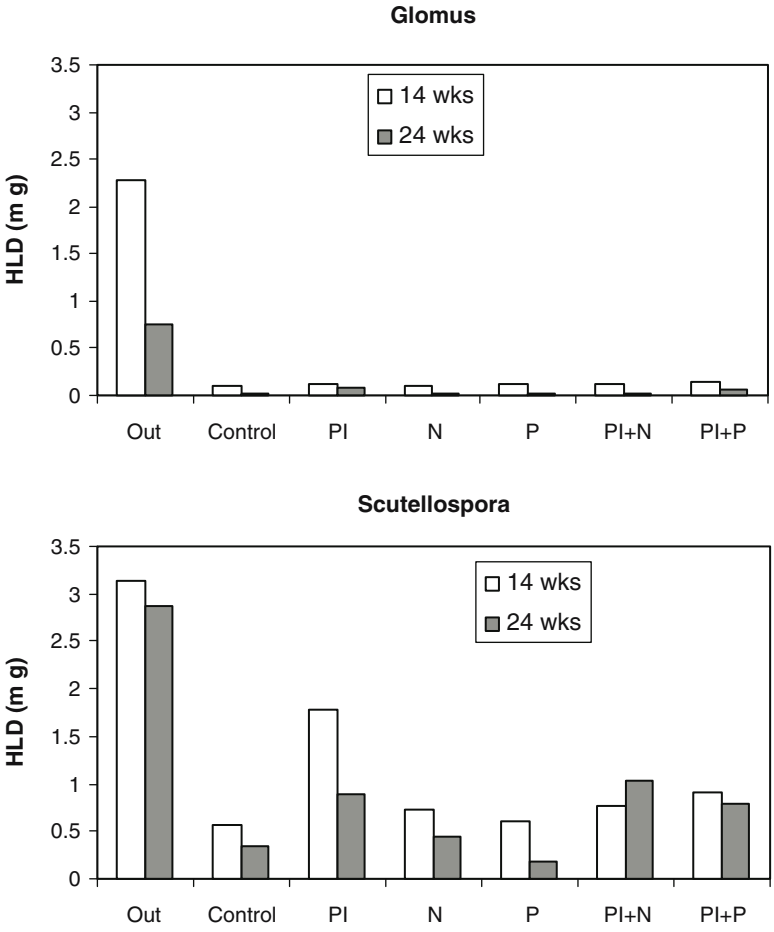


Fig. 4 Multidimensional model. The extraradical mycelium of *Glomus intraradices* and *Scutellospora calospora* was established in the pots for 11 or 21 weeks before introducing the bags with choices and measured 25 days after the choices had been introduced. Hyphal length density values in meters per gram soil at the time of harvest (14 weeks for the "young" mycelium or 24 weeks for the "old" mycelium. *Out* Sand surrounding the mesh bags as a reference, *control* unamended, *P* phosphorus, *N* nitrogen, *Pl* plant, *Pl+N* plant plus nitrogen, *Pl+P* plant plus phosphorus. From Gavito and Olsson (2008) redrawn with permission from Applied Soil Ecology

rather difficult. According to these results, the AMF isolates were mainly looking for a new source of C in a host plant, and *S. calospora* was searching more actively than *G. intraradices*.

The two models have high potential to convey very meaningful information concerning foraging strategies in AMF. They have been used very little and there are still many questions to answer in this topic.

5 Main Challenges and New Approaches in Foraging Studies

Stable and radioactive isotopes have high potential in fungal foraging studies. Tracking the movement of resources is essential to understand the response of the mycelium to resource variability in the environment. AMF foraging differs from plant foraging in the sense that roots are mostly vegetative structures with the only function of absorbing and transporting water and nutrients, whereas AMF mycelium performs both vegetative and reproductive functions. Resources in the extraradical AMF mycelium are therefore shared between both functions, and allocation is a trade-off between growth and persistence. This means that AMF are more similar to whole plants than to roots only. In plants, root to shoot ratios and leaf, flower and fruit proportional biomass can be used as indicators of resource allocation. Movement of resources between these pools shows what the plant is doing. In AMF, a spore/mycelium ratio would not be an adequate measure of C allocation because mycelium has both vegetative and reproductive functions. This implies that the only way to know what AMF are doing is through direct measurements of resource flow within mycelium and spores, and this can only be done by marking the resource in question with an isotope and following the label to a signature molecule like a fatty acid (Boschker and Middelburg 2002; Treonis et al. 2004; Evershed et al. 2006). The incorporation of photosynthesis assimilated ^{13}C into the signature fatty acid 16:1ω5 as a biomarker of AMF (Olsson and Johansen 2000) has been a useful measurement in several experiments. ^{13}C enrichment in the neutral lipid fatty acid 16:1ω5 was positively correlated with ^{13}C enrichment in total C, and this relationship was not found in a biomarker for other fungi, indicating preferential incorporation of C to the AMF biomarker (Olsson et al. 2005).

A recent methodological approach based on stable isotopes is the incorporation of ^{13}C to DNA or RNA (stable isotope probing, SIP). This method gives the advantage of distinguishing the enrichment in different AMF species in a sample (Whiteley et al. 2006), which is not possible with the fatty acid method (Treonis et al. 2004). The fatty acid method is more reliable though in terms of accurate quantification of ^{13}C incorporation. It is cheaper and does not require such strong labeling as needed for gradient centrifugation of DNA or RNA (Wellington et al. 2003). Long-pulse labeling allowed Rangel-Castro et al. (2005) to get enough label to study the influence of liming on microbial communities in grassland soils, but they failed to detect labeled mycorrhizal fungal sequences (expected to show in ^{13}C RNA). SIP has not been used in studies specifically investigating mycorrhizal symbionts and needs to be improved to solve insufficient labeling and primer bias, but has a large potential in AMF foraging studies since species identification is critical for testing functional variation. Extraradical mycelial networks in AMF species are diverse under several criteria (Avio et al. 2006), and are usually intermingled in soil. The main challenge to improve our resolution in foraging studies is the accurate identification and quantification of multiple interacting AMF mycelia in soil.

6 Conclusions

The process of searching for resources is critical for the performance of all organisms, but foraging studies in AMF are still to scarce in the literature. Recent evidence suggests AMF mycelial networks might be highly variable in growth and functional traits that make them weak or strong under certain conditions. Also, some AMF seem more active in foraging than others. Understanding why an intensive foraging activity is advantageous for some isolates is fundamental for our basic knowledge of how AMF interact with their environment and how AMF communities are established.

References

Albertsen A, Ravnskov S, Green H, Jensen DF, Larsen J (2006) Interactions between the external mycelium of the mycorrrhizal fungus *Glomus intraradices* and other soil microorganisms as affected by organic matter. Soil Biol Biochem 38:1008–1014

Augé RM (2001) Water relations, drought and vesicular-arbuscular symbiosis. Mycorrhiza 11:3–42

Avio L, Pellegrino E, Bonari E, Giovantetti M (2006) Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. New Phytol 172:347–357

Bago B (2000) Putative sites for nutrient uptake in arbuscular mycorrhizal fungi. Plant Soil 226:263–274

Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–957

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bago B, Pfeffer PE, Abubaker J, Jun J, Allen JW, Brouillette J, Douds DD, Lammers PJ, Shachar–Hill Y (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. Plant Physiol 131:1496–1507

Bell AD (1984) Dynamic morphology: a contribution to plant ecology. In: Dirzo R, Sarukhan J (eds) Perspectives on plant population ecology. Sinauer, Sunderland, Mass., pp 48–65

Bending GD, Read DJ (1995) The structure and function of the vegetative mycelium of ectomycorrhizal plants. V. The foraging behaviour of ectomycorrhizal mycelium and the translocation of nutrients from exploited organic matter. New Phytol 130:401–409

Boddy L (1999) Saprotrophic cord-forming fungi: meeting the challenge of heterogeneous environments. Mycologia 91:13–32

Boschker HTS, Middelburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. FEMS Microbiol Ecol 40:85–95

Cole ES, Mahall BE (2006) A test for hydrotropic behavior by roots of two coastal dune shrubs. New Phytol 172:358–368

Cui M, Caldwell MM (1996a) Facilitation of plant phosphate acquisition by arbuscular mycorrhizas from enriched soil patches. I. Roots and hyphae exploiting the same soil volume. New Phytol 133:453–460

Cui M, Caldwell MM. 1996b. Facilitation of plant phosphate acquisition by arbuscular mycorrhizas from enriched soil patches. II. Hyphae exploiting root-free soil. New Phytol 133:461–467

De Kroon H, Mommer L (2006) Root foraging theory put to the test. Trends Ecol Evol 21:113–116

De la Providencia IE, Fernández F, Declerck S (2006) Hyphal healing mechanism in the arbuscular mycorrhizal fungi Scutellospora reticulata and Glomus clarum differs in response to severe physical stress. FEMS Microbiol Lett 268:120–125

De Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of Scutellospora reticulata in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Dodd JC, Boddington C, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detaction. Plant Soil 226:131–151

Donnelly DP, Boddy L (1997) Development of mycelial systems of Stropharia caerulea and Phanerochaete velutina on soil: effect of temperature and water potential. Mycol Res 101:705–713

Donnelly DP, Boddy L, Leake J (2004) Development, persistence and regeneration of foraging ectomycorrhizal mycelial systems in soil microcosms. Mycorrhiza 14:37–45

Eissenstat DM, Graham JH, Syvertsen JP, Drouillard DL (1993) Carbon economy of sour orange in relation to mycorrhizal colonization and phosphorus status. Ann Bot 71:1–10

Evershed RP, Crossan ZM, Bull ID, Mottram H, Dungait JA J, Maxfield PJ, Brennand EL (2006) ^{13}C-labelling of lipids to investigate microbial communities in the environment. Curr Op Biotechnol 17:72–82

Farley RA, Fitter AH (1999) The responses of seven co-occurring woodland herbaceous perennials to localized nutrient-rich patches. J Ecol 87:849–859

Farrar JF, Jones DL (2000) The control of carbon acquisition by roots. New Phytol 147:43–53

Fitter AH, Heinemeyer A, Staddon PL (2000) The impact of elevated CO_2 and global climate change on arbuscular mycorrhizas: a mycocentric approach. New Phytol 14:179–187

Friese CF, Allen MF (1991) The spread of VA mycorrhizal fungal hyphae in the soil: inoculum types and external hyphae architecture. Mycologia 83:409–418

Gavito ME, Olsson PA (2003) Allocation of plant carbon to foraging and storage in arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 45:181–187

Gavito ME, Olsson PA (2008) Foraging strategies of the external mycelium of the arbuscular mycorrhizal fungi *Glomus intraradices* and *Scutellospora calospora*. Appl Soil Ecol 39:282–290

Gavito, ME, Bruhn D, Jakobsen I (2002) Phosphorus uptake by arbuscular mycorrhizal hyphae does not increase when the host plant grows under atmospheric CO_2 enrichment. New Phytol 154:751–760

Gill RA, Jackson RB (2000) Global patterns of root turnover for terrestrial ecosystems. New Phytol 147:13–31

Giovanetti M, Sbrana C, Logi C (2000) Microchambers and video-enhanced light microscopy for monitoring cellular events in living hyphae of arbuscular mycorrhizal fungi. Plant Soil 226:153–159

Grime JP, Mackey JML (2002) The role of plasticity in resource capture by plants. Evol Ecol 16:299–307

Gryndler M, Larsen J, Hršelová H, Řezáčová V, Gryndlerová H, Kubát J (2006) Organic and mineral fertilization, respectively, increase and decrease the development of external mycelium of arbuscular mycorrhizal fungi in a long-term field experiment. Mycorrhiza 16:159–166

Harris M, Boddy L (2005) Nutrient movement and mycelial reorganization in established systems of *Phanerochaete velutina*, following arrival of colonized wood resources. Microbial Ecol 50:141–151

Hodge A (2001) Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine patches in soil. New Phytol 151:725–734

Hodge A (2005) Plastic plants and patchy soils. J Exp Bot 57:401–411

Hodge A, Stewart J, Robinson D, Griffiths BS, Fitter AH (1999) Plant, soil fauna and microbial responses to N-rich organic patches of contrasting temporal availability. Soil Biol Biochem 31:1517–1530

Hodge A, Robinson D, Fitter AH (2000a) An arbuscular mycorrhizal inoculum enhances root proliferation in, but not nitrogen capture from nutrient-rich patches in soil. New Phytol 145:575–584

Hodge A, Stewart J, Robinson D, Griffiths BS, Fitter AH (2000b) Competition between roots and soil microorganisms from nitrogen-rich patches of varying complexity. J Ecol 88:150–164.

Jakobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated to *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow into roots. New Phytol 120:371–380

Jansen C, van Kempen MML, Bogemann GM, Bouma TJ, de Kroon H (2006) Limited costs of wrong root placement in *Rumex palustris* in heterogeneous soils. New Phytol 171:117–126

Jentschke G, Godbold DL, Brandes B (2001) Nitrogen limitation in mycorrhizal Norway spruce (*Picea abies*) seedling induced mycelial foraging for ammonium: implications for Ca and Mg uptake. Plant Soil 234:109–117

Joner EJ, Jakobsen I (1995) Growth and extracellular phosphatase activity of arbuscular mycorrhizal hyphae as influenced by soil organic matter. Soil Biol Biochem 27:1153–1159

Kelly CK (1990) Plant foraging: a marginal value model and coiling response in *Cuscuta subinclusa*. Ecology 71:1916–1925

Kembel SW, Cahill Jr JF, Westoby M, Losos JB (2005) Plant phenotypic plasticity belowground: a phylogenetic perspective on root foraging trade-offs. Am Nat 166:216–230

Larsen J, Jakobsen I (1996) Interactions between a mycophagous collembola, dry yeast and external mycelium of an arbuscular mycorrhizal fungus. Mycorrhiza 6:259–264

Lindahl B, Stenlid J, Finlay R (2001) Effects of resource availability on mycelial interactions and ^{32}P-transfer between a saprotrophic and an ectomycorrhizal fungus in soil microcosms. FEMS Microbiol Ecol 38:43–52

MacArthur RH, Pianka ER (1966) On optimal use of a patchy environment. Am Nat 100: 603–609

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London

Marulanda A, Azcón R, Ruiz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungi isolates to water uptake by *Lactuca sativa* L. plants under drought stress. Physiol Plant 119:526–533

Mihail JD, Bruhn JN (2005) Foraging behaviour of *Armillaria* rhizomorph systems. Mycol Res 109:1195–1207

Nakano A, Takahashi K, Kimura M (1999) The carbon origin of arbuscular mycorrhizal fungi estimated from δ^{13}C values of individual spores. Mycorrhiza 9:41–47

Nakano-Hylander A, Olsson P A (2007) Carbon allocation in arbuscular mycorrhizal fungi during colonisation of plant seedlings. Soil Biol Biochem 39:1450–1458

Olsson PA, Johansen A (2000) Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycol Res 104:429–434

Olsson PA, Jakobsen I, Wallander H (2002) Foraging and resourceallocation strategies of mycorrhizal fungi in a patchy environment. In: Van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 93–115

Olsson PA, van Aarle IM, Gavito, ME, Bengtson P, Bengtsson G (2005) ^{13}C-incorporation into signature fatty acids as a way to trace and quantify carbon allocation in arbuscular mycorrhizas. Appl Environ Microbiol 71:2592–2599

Peng S, Eissenstat DM, Graham JH, Williams K, Hodge NC (1993) Growth depression in mycorrhizal citrus at high-phosphorus supply. Analysis of carbon cost. Plant Physiol 101:1063–1071

Pfeffer PE, Douds DD, Bücking H, Schwartz DP, Shachar-Hill Y (2004) The fungus does not transfer carbon to or between roots in an arbuscular mycorrhizal symbiosis. New Phytol 163:617–627

Pritchard SG, Rogers HH (2000) Spatial and temporal development of crop roots in CO_2-enriched environments. New Phytol 147:55–71

Rangel-Castro JI, Killham K, Ostle N, Nicol GW, Anderson IC, Scrimgeour CM, Ineson P, Meharg A, Prosser JI (2005) Stable isotope probing analysis of the influence of liing o root exudate utilization by soil microorganisms. Environ Microbiol 7:828–838

Ravnskov S, Larsen J, Olsson PA, Jakobsen I (1999) Effects of various organic compounds on growth and phosphorus uptake of an arbuscular mycorrhizal fungus. New Phytol 141:517–524

Ritz K, Millar SM, Crawford J W (1996) Detailed visualization of hyphal distribution in fungal mycelia growing in heterogeneous nutritional environments. J Microbiol Methods 25:23–28

Ruiz-Lozano JM (2003) Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13:309–317

Ruiz-Lozano JM, Porcel R, Aroca R (2006) Does the enhanced tolerance of arbuscular mycorrhizal plants to water stress deficit involve modulation of drought-induced plant genes? New Phytol 171:693–698

Schmid B, Harper JL (1985) Clonal growth in grassland perennials. I. Density and pattern dependent competition between plants with different growth forms. J Ecol 73:793–808

Smith SE, Read D J (1997) Mycorrhizal symbiosis. Academic Press, London

St. John TV, Coleman DC, Reid CPP (1983a) Growth and spatial distribution of nutrient-absorbing organs: selective exploitation of soil heterogeneity. Plant Soil 71:487–493

St. John TV, Coleman DC, Reid CPP (1983b) Association of vesicular-arbuscular mycorrhizal hyphae with soil organic particles. Ecology 64:957–959

Stephens DW, Krebs JR (1986) Foraging theory. Princeton University Press, Princeton

Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1997) Clonal growth traits of two *Prunella* species are determined by co-occurring arbuscular mycorrhizal fungi from a calcareous grassland. J Ecol 85:181–191

Tibbett M (2000) Roots, foraging and the exploitation of soil nutrient patches: the role of mycorrhizal symbiosis. Funct Ecol 14:397–399

Treonis AM, Ostle NJ, Stott AW, Primrose R, Grayston SJ, Ineson P (2004) Identification of groups of metabolically-active rhizosphere microorganisms by stable isotope probing of PLFAs. Soil Biol Biochem 36:533–537

Voets L, De la Providencia IE, Declerck S (2006) Glomeraceae and Gigasporaceae differ in their ability to form hyphal networks. New Phytol 172:185–188

Walk TC, Jaramillo R, Lynch JP (2006) Architectural trade offs between adventitious and basal roots for phosphorus acquisition. Plant Soil 279:347–366

Wallander H, Lindahl B, Nilsoon LO (2006) Limited transfer of nitrogen between wood decomposing and ectomycorrhizal mycelia when studied in the field. Mycorrhiza 16:213–217

Wang LX, Mou PP, Jones RH (2006) Nutrient foraging via physiological and morphological plasticity in three plant species. Can J For Res 36:164–173.

Wellington EH, Berry A, Krsek M (2003) Resolving functional diversity in relation to microbial community structure in soil: exploiting genomics and stable isotope probing. Curr Op Microbiol 6:295–301

Whiteley AS, Manefield M, Lueders T (2006) Unlocking the ‘microbial box’ using RNA-based stable isotope probing technologies. Curr Op Biotechnol 17:67–71

Yang ZJ, Midmore DJ (2005) Modelling plant resource allocation and growth partitioning in response to environmental heterogeneity. Ecol Model 181:59–77

Global Diversity Patterns of Arbuscular Mycorrhizal Fungi–Community Composition and Links with Functionality

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1 Introduction

Arbuscular mycorrhizal fungi (AMF, phylum Glomeromycota) are ubiquitous plant root symbionts that have been considered as 'keystone mutualists' in terrestrial ecosystems. They form a link between biotic and abiotic components of ecosystems via carbon and nutrient fluxes that pass between plants and fungi in the soil (O'Neill et al. 1991). The global carbon flux from plants to AM fungi has been estimated as 5 billion tons per year (Bago et al. 2000). The global biomass of the Glomeromycota has been estimated as 1.4 Pg (Treseder and Cross 2006), compared with the estimation of global soil microbial biomass of 13.9 Pg (Wardle 1992). Thus, Glomeromycota is a considerable but largely overlooked sink in the carbon cycle, whilst these fungi possess ecologically important functions in plant nutrition, pathogen resistance and water relations (Smith and Read 1997). A formerly prevailing understanding that all AM fungi are functionally similar is under question and is being replaced by more complex view including gradients of host selection/preference and variation in AM fungal life strategies with evidence of generalists and specialists, ruderals, K- and r-strategists (Helgason et al. 2007; Mathimaran et al. 2007).

AM fungal communities have been shown to affect plant diversity and productivity (van der Heijden et al. 1998; van der Heijden and Sanders 2002). Large differences in functional complementarities between plant and AM fungal species coexisting in the same ecosystem have been demonstrated with plant species from temperate forest (Helgason et al. 2002) and temperate grassland (Klironomos 2003). There is further evidence that plant–AM fungal coupling is of importance for the performance of not only the symbiotic partners but also for ecosystem function (van der Heijden et al. 2006). However, information on spatiotemporal organisation of AM fungal assemblages is still rather limited at all scales. What is the structure of an AM fungal assemblage within a root system? Within a patch of soil? Within a

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patch of a grassland/forest/desert etc.? Among and between different ecosystems, between biomes, climatic zones, continents? Such biogeographical data exist for most of the larger organisms, at least to a certain degree, but they are lacking for microorganisms. It is important to gain a better understanding of AM fungal diversity at a range of scales in order to be able to extrapolate from research with individual isolates and species to functioning of communities and ecosystems. However, knowledge of taxon diversity without a concomitant understanding of the biology of taxa would restrict any understanding of natural patterns.

Methodological obstacles and lack of visibility due to the soil habitat have contributed to the outlined lack of information. Recently, the necessity for more biogeographic information regarding the Glomeromycota has been articulated (Schwartz et al. 2006; Lilleskov and Parrent 2007; Kiers and van der Heijden 2006).

Why do we need to know and understand the natural (taxonomic and functional) diversity patterns of AM fungi? Probably most of what is known about AM fungi in relation to their natural occurrence, physiology, life cycle characteristics, etc., originates from the study of ruderal or 'weedy' species and/or isolates as they are likely to produce (more) spores in nature, are (more) easily cultivable under the greenhouse or laboratory condition and/or grow in economically important ecosystems. However, what we do not know is how widespread these fungi are in nature, how common are their functional traits among other AM fungi, and what other functions or life history strategies are present but unstudied. Survey data gathered during the last decade applying molecular detection and identification methods on AM fungi have demonstrated the common occurrence of presently unidentifiable Glomeromycota fungi in the natural environment. For these, we know nothing of their life style, physiology, or effects on ecosystem functioning. The importance of AM fungi in ecosystem functioning is well established, but the information about the variety of form and function present in nature is clearly insufficient.

In this chapter, we aim to provide an overview about what is known about AM fungal natural (taxonomic) diversity patterns without attempting to cite every relevant publication. We discuss taxon delimitation and methodological issues affecting the revealed diversity and its interpretation. We outline the evidence of functional diversity and ways to link it with taxonomic diversity patterns in nature. Finally, we outline the areas and directions of research in the field of AM fungal ecology we consider essential to explore with fresh or renewed vigor in order to advance our understanding of functioning of AM fungi in ecosystems in the changing world.

2 Taxon Diversity of AM Fungal Communities

2.1 Taxon Delimitation

In order to estimate the number of taxa in a sample, one needs first to delimit them. Principles of species delimitation of Glomeromycota are far from solved, though traditionally a morphological species concept has been used. The suitability and

possible pitfalls of using rDNA sequences for molecular identification purposes are similarly disputed. However, this problem is beyond the scope of the present chapter; for recent treatises on the topic the reader is referred to Clapp et al. (2002), Redecker et al. (2003), and Redecker and Raab (2006).

In the present chapter we use phylogenetically defined groups (of mostly rDNA sequences) as operational taxonomic units (OTU) if fungi were identified by molecular means. These groups have been intended to correspond approximately to species level. We refer to these groups as 'taxa', because the relationship between morphologically defined AM fungal species and phylogenetically defined OTUs of sequences of environmental origin is unclear due, primarily, to the scarce information about intraspecific genetic variability of the Glomeromycota. In the literature the terms 'sequence group' and 'sequence type' are commonly used. The level of sequence difference between these taxa can be 1–3% depending on the researcher's choice (99–97% similarity; e.g., Helgason et al. 1999). Still, a combination of roughly fixed similarity level with good support of phylogenetic clades might be preferred as a basis for OTU definition over the arbitrary sequence similarity level. The sequence divergence in the SSU rDNA region in particular might be insufficient in order to separate some taxa, as argued for example in the *Glomus intraradices* group, where other genes might be more appropriate (Raab et al. 2005). Overall, in order to be more able to efficiently detect and identify naturally occurring Glomeromycota, there is a clear need to clarify Glomeromycota taxonomy linking molecular and morphological diversity. We need to gain a concerted understanding of intra- and interspecific variation in morphology, genetics, development, and physiology of these fungi. Developments in this area would greatly enhance our ability to interpret ecological data, but also to learn about economically important AM fungi and provide grounds for extrapolating from individual studies.

At the global scale, the number of described Glomeromycota species is over 150 (Walker and Trappe 1993). The number of SSU rDNA sequence-based taxa of Glomeromycota was ca. 100 at the end of the year 2005 (Öpik et al. 2006b). A minority of these taxa are related to morphotaxa, whilst many others are known only as sequences from a single location. The 'sequence-only' taxa are increasingly becoming known from several locations due to the increasing number of molecular surveys of natural Glomeromycota. However, the proportion of matches with 'known' species is likely to increase as more isolates of culture collections are sequenced; presently only a small number are represented in sequence databases. Therefore, the total number of Glomeromycota is probably much greater than the abovementioned 150 species, as previously proposed by Helgason et al. (2002).

2.2 Diversity of AM Fungi in Soil as Spores

As with other organisms, the taxon richness of AM fungi has been shown to vary from location to location. There is a wealth of spore-based AM fungal surveys from a range of locations and ecosystems. Traditional methods to identify AM

fungi have for a long time been based on spores captured from soil, either by direct extraction or via so-called 'trap-culturing' with plants (e.g., Stutz and Morton 1996). The value of spore surveys has been questioned and re-valued (see Sanders 2004). Most importantly, it is still unknown what triggers the onset of sporulation, what determines sporulation intensity and which factors influence observed variation in spore numbers of AM fungi in natural environments. Furthermore, the communities of spores in the soil and fungi colonizing roots are not necessarily identical (Clapp et al. 1995); neither is there a direct relationship between sporulation and root colonization levels (see Dodd et al. 2000). Therefore, while spores identified from soils constitute important information regarding the species pool with a potential to colonize plant roots in a given location, as well as revealing some biological and functional properties of morphospecies, spore communities should not be equated with the total AM fungal community at a site without further information about root-colonizing fungi. Soil spores could be viewed as part of the bank of propagules of AM fungi in soil, analogous to the seed bank (see below).

Probably the highest known AM fungal richness at a single site, 37 taxa, was detected by extensive trap-culturing of spores over many years from an old field (Bever et al. 2001). The diversity patterns of Glomeromycota spore communities are thoroughly reviewed by Chaudhary and Johnson in this volume.

2.3 Diversity of AM Fungi in Roots

Pioneering publications of Simon et al. (1992) and Helgason et al. (1998), who first described a molecular method to detect and identify Glomeromycota in plant roots, and who designed the present most commonly used AMF-specific primer, respectively, were followed by a large increase in information about natural root-colonizing AM fungal communities. Described natural diversity patterns include community composition differences in space and time (Helgason et al. 1999; Daniell et al. 2001), between hosts (Helgason et al. 2002; Vandenkoornhuyse et al. 2002), and between management practices (Helgason et al. 1998; Hijri et al. 2006; Öpik et al. 2006b), within a root system (Scheublin et al. 2004).

2.3.1 Richness

The number of AM fungi inhabiting a single root system has been shown to reach ca. 10 taxa (e.g., Helgason et al. 2007) and can possibly be greater. Evidence relating to plant individual-level AM fungal richness is rather limited. During the life of an individual plant some changes probably occur in terms of number of AMF taxa inhabiting the roots. There are some indications that seedlings may be colonized by more and different set of AM fungi as compared to adult plants (Husband et al. 2002b; Öpik et al. 2003).

We are aware of very few studies where the variability of AM fungal assemblages has been studied within a root system. A study of *Viola mirabilis* and *V. elatior* from different locations showed that replicate 1-cm root samples from the same plant individual hosted on average 3–4 AM fungal taxa and a single plant 7–8 taxa (Öpik et al. 2006a). Similarly, in metal-contaminated or arable field locations, typically one or two fungal types have been detected in 0.5- to 1-cm root fragments, whilst the total taxon richness in the sites could be five or more taxa (Jacquot-Plumey et al. 2001; Kjøller and Rosendahl 2001; Turnau et al. 2001).

The number of AM fungal taxa per host plant species at any location may differ between habitat types (Öpik et al. 2006b). Currently the highest AM fungal richness has been observed in tropical forests (18 SSU rDNA taxa of AMF/plant species), followed by grasslands (8), temperate forests (5.6) and habitats under anthropogenic influence (arable fields and polluted sites, 5.2; Öpik et al. 2006b).

Furthermore, ecosystems vary in the number of fungal species identified from them: 23 AM fungal sequence types have been identified from a single temperate broad-leaved forest location, 10–24 from different temperate grassland locations, 7–22 from dry or wet tropical forests, 2–7 from temperate arable fields, 5–7 in few studied polluted sites, and 14–20 from temperate wetlands (references in Öpik et al. 2006b; also Franke et al. 2006; Helgason et al. 2007; Hijri et al. 2006; Santos et al. 2006; Vallino et al. 2006; Wubet et al. 2006). Care need to be taken in the interpretation of such data as sampling intensity has not been even across different biomes and studies. However, related evidence suggests that there are trends of different AM fungal species richness in different ecosystems.

Again, little is known about the number of species in the Glomeromycota flora of different regions. Species lists of AM fungi have been compiled for only a few locations (Błaszkowski 1994; Tadych and Błaszkowski 2000). An important resource to establish regional (and ecological) information about Glomeromycota flora are the databases of culture collections such as the International Bank of the Glomeromycota (BEG, http://www.kent.ac.uk/bio/beg) and the International Culture Collection of (Vesicular) Arbuscular Mycorrrhizal Fungi (INVAM, http://invam.caf.wvu.edu/cultures/accessions.htm) which provide information on location, habitat type, edaphic characteristics, host plant species etc of the fungal isolates maintained in these collections. For more thoroughly investigated regions this data would provide species lists and distribution data which, though limited to cultured Glomeromycota, give an estimate of richness patterns within these regions.

The number of AMF taxa discovered in a location might be an indicator of ecosystem status in terms of disturbance intensity versus stability. However, in terms of ecosystem function the phylogenetic taxon distribution might be of greater importance because larger families of Glomeromycota exhibit some conserved functional trait diversity (Maherali and Klironomos 2007). Therefore, effects of AMF diversity (including richness) on ecosystem function may be more obvious when fungi from different genera/families with different strategies are present, as argued by van der Heijden et al. (2006). Thus, the composition of the AM fungal community can be more important than number of taxa.

2.3.2 Composition

Glomeromycota taxon distribution patterns within a community, expressed as diversity indices, vary remarkably between sites/ecosystems and across seasons, demonstrating different levels of taxon dominance. For example, the Shannon-Wiener diversity index (*H*) of AM fungal communities may vary from <1 in some arable fields (Daniell et al. 2001; Hijri et al. 2006) to >2 in tropical forests (Husband et al. 2002a; Wubet et al. 2004) and temperate wetlands (Wirsel 2004; and see Table 1 in Öpik et al. 2006b).

At the level of individual plants, root-colonization data suggest that several different AM fungi can colonize a root fragment and that they occupy different spatial niches within a root system (e.g., Merryweather and Fitter 1998a). *Viola mirabilis* and *V. elatior* individuals, from different locations, hosted different AM fungi in replicate 1-cm root samples from the same plant (Öpik et al. 2006a). However, when the entire root system was considered, the fungal assemblages showed plant species-related differences regardless of the location of origin (Öpik et al. 2006a). A pot experiment with *Medicago truncatula* co-inoculated with *Gigaspora rosea*, *Glomus mosseae* and *Glomus intraradices* demonstrated different frequencies (occurrence) of each constituent species in 1-cm-long root fragments (Jacquot-Plumey et al. 2001). Furthermore, there is evidence that the bulk roots and nodules of legumes may be inhabited by distinct AM fungal assemblages (Scheublin and van der Heijden 2006).

A (molecular) quantitative approach based on real-time PCR was taken by Alkan et al. (2006) who reported different spatial patterns of root colonization by *Glomus mosseae* and *G. intraradices* in various experimental settings including phosphorus availability gradients and salinity stress. These observations were based on one plant individual per treatment, and one isolate per fungal species. Whilst quantitative information regarding the AM fungal colonization is badly needed, this report highlights the requirement of the quantitative PCR methodology to generate sample throughput high enough to allow robust testing of hypotheses. The lack of replication of experimental units provides no confirmation on consistency or significance of the reported differences between experimental treatments, including different isolates and the studied species.

Small-scale spatial arrangement of AM fungal taxa has rarely been described. Soil spore data suggests, however, that at a local scale the AM fungi can occur in a highly spatially structured, i.e. patchy way (Abbott and Robson 1991; Allen 1996; unpublished data in Hart and Klironomos 2002; Klironomos et al. 1999). This was recently corroborated by Wolfe et al. (2007) who sampled a single 2 × 2 m plot and discovered considerable variation in T-RF richness of AM fungi in roots. In addition, plant species affects the sporulation intensity (total spore number) and spore diversity in a grassland (Johnson et al. 1992). Seasonal colonization patterns and variability between different years in terms of *Glomus* and *Scutellospora* morphotypes colonizing bluebell roots was described by Merryweather and Fitter (1998b). Temporal changes in root-colonising community structure were reported in arable field rotation (Daniell et al. 2001) and in broad-leaved forest understorey (Helgason et al. 1999).

A fine-scale spatial description of root-colonizing AM fungal communities demonstrated variable relative abundance of 5 RFLP types in adjacent 2 × 2 m plots (Yamato and Iwase 2005). Similarly fine-scale survey of AM fungi was performed using bait-plants grown on soil cores from a single 2 × 2 m plot in a calcareous fen. High spatial variation in AM fungal T-RF richness was revealed, ranging from 2 to 14 T-RFs per soil core, but no significant spatial patterns or association with the aboveground plant community pattern were detected (Wolfe et al. 2007). Clearly more descriptive data are needed about small-scale spatial and temporal arrangement of communities of AM fungi, considering both intra- and extraradical fungi.

At the ecosystem or biome level there are indications that specific AM fungal communities, of differing compositions, occur in broadly defined habitat types such as tropical forests, temperate forests, disturbed habitats or grasslands (Öpik et al. 2006b).

In conclusion, descriptive evidence on natural AM fungal diversity is accumulating. However, interpretation of the emerging patterns is severely hampered by the lack of knowledge regarding functional trait diversity, by ambiguities in Glomeromycota taxonomy and by poor comparability of individual studies.

3 Methodological Issues, Obstacles

3.1 Effect of Sampling Design on the Observed Diversity Patterns: Importance of Replication

Sampling design can understandably affect the diversity and structure of the AM fungal assemblages observed in samples. It is important to consider the source of data when drawing conclusions based on molecular root-colonizing AM fungal surveys.

The molecular identification of AM fungi in the natural environment is frequently done by extracting the DNA from a root (or soil) sample followed by PCR (single-step, nested or several group-specific PCR-s), separation of multiple products by cloning or electrophoresis (SSCP, DGGE, TGGE, or T-RFLP after digestion), and sequencing clones or individual bands. Size of root samples (as published in papers) ranges from 0.5 cm to ca. 20 cm. The samples may be collected following different spatial/temporal sampling designs. The size of soil samples taken depends on the DNA extraction method, but usually does not exceed a 1-g representative sample from a larger initial homogenous sample. It is worthy of contemplation how well these small samples represent AM fungal (or other soil microorganism) communities in natural ecosystems. Can a forest be described with a handful of pieces of root?

It is possible to estimate the plant individual-based fungal community structure by studying several small root samples, instead of one large sample, from the same plant. This has rarely been done, but such an approach reveals the spatial structure of the fungal community within root system (see above; Jacquot-Plumey et al. 2001;

Kjøller and Rosendahl 2001; Turnau et al. 2001; Öpik et al. 2006a). Commonly in published papers, if the size of a sample is specified, ca. 20 cm length of roots from an individual plant constitutes a sample, thus also retaining the information of the host identity. However, depending on the research question and the spatial scale of interest, different levels of sample pooling down to a single sample may be considered as an alternative to intensive sampling. In order to compare fungal assemblages of locations, but eliminating variation related to host-specificity or preference, random root samples consisting of a mixture of several plant species might be used (e.g. Heinemeyer et al. 2004). This approach would require that plant species are roughly similarly distributed in the study sites in order to avoid hidden effect of host-preference due to different representation of plant hosts in samples. However, if host effect to AM fungal diversity is high and host distribution in samples uneven, this may result in hidden host effect on the recovered fungal diversity.

Some authors have used an approach where individual samples are pooled after collection or after DNA extraction (Jumpponen et al. 2005; Saito et al. 2004), possibly in order to minimize number of samples to be cloned and thus the cloning expense. From such a pooled sample, larger number of clones might be screened and/or sequenced. Is it better to analyse 10 clones from each of 10 samples or 25 clones from each of 4 samples? Assuming that fungal taxa are patchily distributed in the study area but their relative abundances are more or less even, pooling samples before cloning may enable a description of the real diversity whilst screening fewer clones in total. On the contrary, if the study area is dominated by a few taxa, but there are many infrequent taxa, more clones would need to be screened either from pooled or unpooled samples in order to capture most of the taxon diversity present. Furthermore, pooling in this case would reduce the probability of recording rare taxa present compared to an unpooled strategy.

Depending on the expected diversity and spatial agglomeration of taxa in the study system, a different number of clones (in total and/or per sample) would describe the fungal community present in a sample at a level satisfactory for the research question. Rarefaction analysis implemented by Wirsel (2004) suggests that larger number of samples would have detected higher diversity in autumn (over 20 as opposed to 16 discovered OTU-s), but not in spring and summer. Number of clones screened per sample varies widely in publications (see Table 1 in Öpik et al. 2006b). There seems to be a trend that a larger sampling effort (i.e., more samples, ignoring the number of clones screened) reveals a larger number of fungal taxa (Öpik et al. 2006b). This suggests that a ‘satisfactory’ level of sampling intensity has mostly not been reached. Sampling intensity should depend on the study system – how many taxa are in the system and how evenly/unevenly they are distributed? For example, a taxon-poor system like fields in intensive agriculture could be described by a smaller number of samples than a taxon-rich system, e.g. grasslands or tropical forests. However, if a taxon-poor system is heavily dominated by a single taxon, then more samples are needed to recover the other, infrequent taxa. As the recovered diversity might not be a linear function of sampling intensity (Suzuki et al. 2004; see below), the best one can do at the present state of knowledge is to be aware of the effect of sampling and to be careful when drawing conclusions.

Klironomos et al. (1999) suggested that researchers should perform geostatistical analyses to describe the spatial distribution of the organisms/processes coupled with power analyses to assess required sample sizes. Their own results from a 3 × 10 m grassland plot suggest that a fifth of the samples are necessary to describe (with a set target of power = 0.75) abundance of *Acaulospora* spores, and half for fungal biomass, if using stratified sampling as opposed to regular grid sampling. However, this presumes that spatial distribution of variables remains constant, and the abundance hot-spots are known (Klironomos et al. 1999).

Regarding the necessary sampling design for reliably recovering taxon composition we are not aware of any explicit analysis for soil microbial communities though sampling scale and (soil) sample size were discussed by Ramette and Tiedje (2007). Suzuki et al. (2004) investigated the effect of sampling design and intensity on recovered genetic structure of a clonal plant population, dependent on the underlying genetic structure of the population (small or large genets, number of different genets). Their simulation results are also applicable to molecular microbial diversity studies, as a microbial taxon identified by molecular means could be analogous to a genet, and a plant population to microbial community. Suzuki et al. (2004) demonstrated that different sampling strategies (random, grid-based, or belt sampling, hierarchical or not) performed equally effectively in recovering genet number if the actual genet numbers were low, but not when high. Specifically, random sampling returned smaller number of genets, if the genets were small. This may be important to consider in AM fungal studies, where many taxon-rich communities show high dominance with many infrequently occurring taxa. Furthermore, apparent frequency might be confounded by variation in probability of detection due to variable colony size. Also, sampling intensity (number of samples per area) became important at higher genet numbers, the higher intensity strongly positively affecting the recovered diversity of genets (Suzuki et al. 2004). Thus, the overall number of samples, their spatial positioning and size are all potentially affecting the outcome of mycorrhizal diversity surveys. The degree of these sampling inflicted biases remains to be clarified at present.

In conclusion, the spatial structure of AM fungal assemblages at any given location has not been rigorously assessed, and the effect of sampling design on estimated diversity should be considered more carefully in future studies and should be tested as the capacity of sample processing increases.

3.2 Effect of Primers/Marker Region on the Observed Diversity Patterns

There has been a number of primer sets used in molecular surveys of the Glomeromycota. The use of molecular methods expanded greatly after the design of a primer (AM1) that in combination with a general fungal primer NS31, targets the central portion of the nuclear small subunit ribosomal RNA

gene preferentially amplifying AM fungal sequences (Helgason et al. 1998). This primer pair was later shown not to amplify the so-called deeply diverging lineages (Redecker 2000). To circumvent this issue Redecker (2000) designed a range of family-specific primer sets which capture a wider range of AM fungal types but require the application of several individual PCR reactions to describe full Glomeromycota diversity in contrast to the single PCR reaction necessary if using primers AM1/NS31. Group-specific primers for *Glomus mosseae-intraradices* clade (LSURK 4f and 7r) were also introduced by Kjøller and Rosendahl (2000), and have been later used by Kjøller and Rosendahl (2001), Nielsen et al. (2004), and Öpik et al. (2006a). One may argue, that this primer set excludes most of the taxonomic range within Glomeromycota, but the *Glomus* group A and taxa amplified by LSURK 4f/7r have been the most commonly detected group using more general primer sets. Further attempts have been made to find all-inclusive primer pairs by Saito et al. (2004) and Wubet et al. (2006) but the efficiency of these pimers in applications other than those of the authors has yet to be reported.

The issue of comparability of data gathered by independent researchers also deserves more attention. Use of the same marker system in many studies enables accumulation of comparable data, which can later be used in meta-analyses, for compiling distribution maps and so on. Wide use of SSU region (NS31/AM1 primer pair) in AM diversity studies enabled Öpik et al. (2006b) to compare richness and taxon composition of AMF in samples from worldwide locations, revealing biogeographical patterns of SSU rDNA taxa. Other marker regions have not accumulated much comparable data which also hinders interpretation on a wider background.

There can be a substantial effect of DNA marker region on conclusions drawn from the detected diversity. Wubet et al. (2006) report on the occurrence of unique AM fungal sequence types colonizing roots in Afro-montane forests. They present two parallel phylogenetic analyses, one based on full SSU rDNA region, another based on the middle fragment of SSU rRNA gene amplified by the NS31/AM1 primer set. The full length analysis suggested that there is no Glomeromycota species closely related to the detected environmental sequences. However, when analyzing the region amplified by the more commonly applied NS31/AM1 fragment, for which relatively large amounts of database sequences are available, at least three of the reported sequence groups were related to the environmental or spore-derived sequences from earlier studies, including two of the dominant types of the study ecosystem.

Though the actual diversity of AM fungi in the world is probably far higher than our current knowledge, one needs to be aware of the influence of the degree of analysis of the marker region applied when interpreting the results. Probably all publications of AM fungal molecular diversity during the recent years have reported some new sequence groups alongside with the consistently re-occurring ones. It is likely that the groups currently known only from the environment may be present in culture collections, but unsequenced, or may represent fungi resistant to culture.

4 Factors Shaping Communities of AM Fungi

More than a decade ago Abbott and Robson (1991) wrote: "our understanding of the factors that affect the occurrence of VA mycorrhizas is limited and suffers because insufficient is known of the lifestyles of most species of VAM fungi." This is still valid and in the light of molecular evidence on AM fungal occurrence gathered since, the insufficiency of the knowledge about biology of Glomeromycota is at least equally limiting. Here, we consider propagation and dispersal as factors shaping AM fungal communities as an example, but note that environmental characteristics and species interactions, including host limitation, might be of similar importance.

4.1 Dispersal, Propagule Bank

Are AM fungal communities limited by dispersal (in an ecological time-scale)? What is known about the role of recent dispersal versus speciation and tectonic events, ice-ages etc. as factors determining the occurrence of AM fungal taxa at any particular location (in an evolutionary time-scale)? Evidence from ectomycorrhizal colonization of endemic plant species of a basal dipterocarp clade coupled with information about tectonic history and fungal phylogeny has allowed the generation of a hypothesis that the ectomycorrhizal symbiosis may have Gondwanan origin (Alexander 2006). What about the evolutionarily earlier arbuscular mycorrhizal fungi? Recently, dispersal limitation was shown to be an important factor shaping AM fungal community composition, where more distant communities in the same soil type were more dissimilar than proximal assemblages (Lekberg et al. 2007). Soil microbial communities' (dis-) similarity has been shown to be driven by dispersal limitation rather than by environmental heterogeneity (reviewed by Green and Bohannan 2006). However, there is contradicting evidence in other microbial groups suggesting that edaphic variables (particularly soil pH) might be more important than distance (dispersal) in explaining soil bacterial community composition (Fierer and Jackson 2006).

Interpretation of natural diversity patterns should bear in mind the mechanisms by which organisms reach their habitat. In relation to the Glomeromycota, different means of dispersal may be dominant in different ecosystems, but dispersal strategy is likely to vary between species, or higher taxonomic groups. AM fungi propagate and disperse via soil-borne (asexual) spores, mycelial fragments and colonized root pieces (Smith and Read 1997). The large size of the spores (30–700 μm) and their formation in the soil are a reason for their relatively poor dispersal ability (Molina et al. 1992). Dispersal mechanisms include wind, water, and small animals (Smith and Read 1997). Wind is probably more important as a dispersal agent in open, wind-eroded places like deserts or arable fields, when not under crop, whilst small animals may account for most dispersal in closed canopy habitats like grasslands and forests. Allen (1988) has noted that there is limited wind dispersal of AM fungal

spores in humid climates, but good wind dispersal in desert steppe. For example, wind has been shown to disperse mainly small *Glomus* spores, up to a distance of ca. 2 km in a disturbed arid ecosystem (Warner et al. 1987) and rodents can disperse considerable quantities of sporocarp-forming *Glomus* species in a tropical forest (Mangan and Adler 2002).

Considering dispersal as a factor shaping AM fungal communities, one needs to take into account some specific properties of Glomeromycota: the spores may maintain the ability to germinate over several years (McGee et al. 1997); the spores (of *Gigaspora*) may germinate repeatedly before a host plant is encountered (Bago et al. 1998); and spores may exhibit dormancy and thus there can be a pool of viable spores in the soil. Dormancy may also be an important mechanism for synchronizing spore germination with rapid root growth and favourable conditions for colonization in temperate regions (Tommerup 1985). It is likely that the type or occurrence of dormancy will depend on the ecosystem; this is a subject that is worthy of further investigation. Persisting spores, intact mycelium, fragments of hyphae and colonized roots in the soil may be thought of as a 'propagule bank', analogous to the plant seed bank, that is waiting for suitable conditions to germinate and grow and colonize new plant roots.

How would ability to disperse affect the species pool of Glomeromycota? Pärtel and Zobel (2007) proposed that arrival probability, which is a function of dispersal probability, distance from source and landscape permeability, would help to better explain the patterns of plant diversity. Dispersal probability is understood here as a function of number, persistence and dispersability of propagules of a taxon. The same concept would be useful when seeking explanations to Glomeromycota occurrence and diversity. For example, a taxon with many small spores and ability to disperse by mycelial fragments would have larger dispersal ability than a taxon with few large spores and no ability to use mycelium as propagules, whereas a species producing large spores with greater longevity would be more likely to maintain a spore population. Spore size, the number of spores produced, range of available propagule types (spores, colonized roots, and mycelial fragments) are parameters which are known to differ among phylogenetic groups of Glomeromycota. Furthermore, it is currently unknown if the propagation and dispersal characteristics of Glomeromycota are linked with particular biomes or habitat types. It is intuitive to hypothesize that the functional trait diversity of AM fungi is different in different ecosystems. Testing these concepts would greatly enhance our understanding of natural AM fungal diversity patterns.

4.2 Links Between Habitat Type and AM Fungal Distribution Patterns

A recent meta-analysis of global distribution patterns of root-colonizing AM fungi demonstrated that different types of ecosystems may host different assemblages of AM fungi (Öpik et al. 2006b). Some information to test this suggestion would arise

if selected plant species occurring in a range of ecosystems would be investigated in replicated locations in each of the ecosystems. There are limited data of this kind and none from a considerable range of ecosystems: epiparasitic plants in tropical forests from Argentina and French Guyana showed strict specialization for an AM fungus (Bidartondo et al. 2002). Similarly low richness was reported for the liverwort *Marchantia* in a range of locations in New Zealand (Russell and Bulman 2005) and for *Pulsatilla* (Ranunculaceae) plants in Estonian dry Scots pine forests, road verges and grasslands, but the number of samples from each site was small (Öpik et al. 2003). Mummey and colleagues (Mummey et al. 2005; Mummey and Rillig 2006) showed that *Centaurea* invasion changed the AM fungal community composition, thus suggesting a host effect on AM fungal composition. Furthermore, the meta-analysis of Öpik et al. (2006b) could cover just the limited range of ecosystems investigated by comparable means at that time. This included a small selection of world's ecosystems or biomes. Expansion of the habitats studied will hopefully add to the picture in the future allowing a more focused analysis of these questions in time.

5 Linking Taxonomic Diversity with Functionality

If we assume that taxonomic diversity bears some relation to functional diversity, an understanding of the functional traits of community component taxa is required to be able to interpret the taxonomic diversity patterns observed in nature. The present evidence in support of the above relation is not promising, as discussed by van der Heijden et al. (2004). It has also been proposed that instead of taxonomic diversity it would be more informative to register the functional diversity of communities (McGill et al. 2006). In terms of ecosystem function the diversity (and measurement) of functions such as plant growth promotion and nutrient uptake by AM fungi may be more important than taxonomic diversity (Mathimaran et al. 2007). Recently, in a similar vein, van der Heijden and Scheublin (2007) proposed a list of functional traits to be measured for AM fungi. Here, we provide an overview of what is known about functional traits of Glomeromycota, mostly following the trait list of van der Heijden and Scheublin (2007), and draw a functional sketch of some widespread and apparently specialist Glomeromycota species.

5.1 Functional Traits of AM Fungi

For plants, there is a wealth of information about their functional traits including abiotic requirements (Ellenberg et al. 1991), clonal growth traits (CLOPLA, http://clopla.butbn.cas.cz/index.php, Klimes and Klimesova 1999), dispersal traits (Pärtel and Zobel 2007) and others. For the Glomeromycota, as for many microscopic organisms, there is limited information available. For the Glomeromycota this often

relates to their growth characteristics, root colonization and sporulation rate, means of dispersal, host range, requirements for abiotic conditions such as soil pH, nutrient content, temperature, etc. A full set of the above proposed characters (the trait list) is probably present for few species, if any, and under a limited range of conditions. It is recognized that more research should be performed to characterize the ecological attributes of AM fungal species (Schwartz et al. 2006).

5.1.1 Mycelial Growth

Growth of AM hyphae appears not to be directed towards phosphorus source, but towards plant root; thus the prime fungal resource to forage is a host plant (Olsson et al. 2002). Growth rate of AM hyphae (runner hyphae) can exceed 1–3 mm/day in laboratory microcosms (Jakobsen et al. 1992a) and 1.4 mm/day in a sand dune ecosystem (Olsson and Wilhelmsson 2000). Hyphal length density under experimental conditions is ca. 15 m/cm^3 (Jakobsen et al. 1992) and 80–110 m/g in natural grasslands (Miller et al. 1995); it may vary between fungal taxa by as much as 2–30 m/g (Jakobsen et al. 1992b).

Hyphal length and density can be very different between species, genera and families (Avio et al. 2006; de la Providencia et al. 2005), but also within species (Munkvold et al. 2004). Development of monoxenic culture systems allows the description of culture characteristics of Glomeromycota including colony architecture and spore development traits (see Table 1 in Dalpe et al. 2005 for characteristics of presently described species).

Anastomosis formation between hyphae of the same isolate has been demonstrated for *Glomus* (Giovannetti et al. 1999), and more rarely for *Scutellospora* (de Souza and Declerck 2003), *Acaulospora* (Tommerup 1988; after de Souza and Declerck 2003) and *Gigaspora* (de la Providencia et al. 2005) species. Furthermore, anastomosis density was higher in Glomaceae than in Gigasporaceae isolates (de la Providencia et al. 2005).

There is also evidence on different occupation of rhizosphere space by different fungi. Experiments with single isolates of *Glomus intraradices* and *G. mosseae* revealed that the extraradical mycelium of *G. intraradices* grew predominantly near the plant root, whilst that of *G. mosseae* was evenly distributed close to and further away from the root (Drew et al. 2006). Similarly, occupation of different layers of root tissues by different isolates has been reported (Cano and Bago 2005).

5.1.2 Sporulation, Colonization Pattern and Persistence

Spores are the main source of inoculum in the genera *Scutellospora* and *Gigaspora* (Boddington and Dodd 2000; Jasper et al. 1993), where sporulation also needs to be preceded by a prolonged phase of root colonization (Dodd et al. 2000). In contrast, sporulation is not required in order to colonize new roots in the case of many *Glomus* species. Therefore, *Glomus* spores may be relatively infrequent in native

soils, even if root colonization by *Glomus* is abundant (Clapp et al. 1995; Jasper et al. 1991). Colonization of new roots by *Glomus* is preferably started from intact mycelium in the soil, or from hyphal or colonized root fragments (McGee 1989).

Some, but not all, *Glomus* species (e.g., *G. invermaium*) are rather vulnerable to disturbance that disrupts the mycelial network; this is in contrast to *Gigaspora*, *Acaulospora* and some other *Glomus* species (e.g., *G. monosporum*, *G. manihotis*), which may show higher colonization initiation upon disturbance (Braunberger et al. 1996; Boddington and Dodd 2000). However, because Gigasporaceae invest mostly in somatic growth (mycelium) rather than in reproduction in terms of propagule numbers (they have large and few spores), but some *Glomus* taxa invest more in reproduction than somatic growth (many small spores, early onset of sporulation), de Souza et al. (2005) concluded that Gigasporaceae are adapted to live in stable environments.

There is experimental evidence that spore dormancy varies with AM fungal species (reviewed by Juge et al. 2002). However, isolates of *Glomus mosseae* from different geographic locations have shown either no dormancy (Douds and Schenck 1991), or they need storage at low positive (+6°C; Hepper and Smith 1976) or at negative temperatures (−10°C; Safir et al. 1990) in order to break dormancy, indicating adaptation to different climatic conditions.

The mycelium of AM fungi can survive a period of freezing (Addy et al. 1998; Kabir et al. 1997). More interestingly, root colonization by *Glomus* species was shown to be little affected by a simulated winter in contrast to a severe reduction in next-season colonization of both *Scutellospora* and *Acaulospora*; the combination of plant host and fungus species also affected the fungal response to freezing (Klironomos et al. 2001). Thus, freezing tolerance is another important characteristic of AM fungi inhabiting periodically frozen soils and may explain the relative rarity of these *Scutellospora* and *Acaulospora* groups in surveys from the temperate/boreal zone (Öpik et al. 2003; Santos et al. 2006).

5.1.3 Nutrient Uptake and Fungal Carbon Use

Differences in phosphorus uptake efficiency between selected AM fungal isolates from different genera were first shown by Jakobsen et al. (1992a). More recently it has been demonstrated that fungal species may exert different effects on the expression of plant nutrient uptake related genes (Burleigh 2001; Burleigh et al. 2002).

Do growth patterns in soil (distance from roots) reflect substrate use strategies? Smith et al. (2000) provide evidence that *Scutellospora calospora* obtained soil P from close to the root, whilst *Glomus caledonium* acquired P from both close to and further away from the root. In an experiment with three *Glomus* species, Jansa et al. (2005) demonstrated that *Glomus mosseae* and *G. intraradices* acquired P from a greater distance from roots than *G. claroideum*. However, rapid colonization of soil and P uptake from distance did not correlate with a greater net P benefit for the plant.

AM fungi receive all their carbon from the host plant. Different fungal species have been shown to capture different proportions of the host's photosynthetically

fixed carbon (Pearson and Jakobsen 1993). However, mycorrhizal plants can have higher photosynthetic rates compared to non-mycorrhizal plants (Eissenstat et al. 1993).

5.1.4 Interactions – Host Range, Competitive Ability

AM fungi have been shown to differ in their host range (Helgason et al. 2007; Smith and Read 1997). However, more data are needed relating to more species combinations of host and fungus.

Competition between AM fungi was probably first studied by Hepper et al. (1988) who discovered in a pot experiment that a *Glomus* sp. was unable to colonize leek roots if either *Glomus mosseae* or *Glomus caledonium* were present, indicating that fierce competition was taking place during the phase of root colonization. A similar result was obtained by Pearson et al. (1993) who showed that increasing inoculum levels of a *Scutellospora* isolate were negatively correlated with the rate and extent of *Glomus* isolate colonization. Later, in vitro experiments have been carried out to investigate competition between co-occurring AM fungal isolates. Quantitative mapping of extraradical mycelial growth and sporulation of *Glomus* and *Gigaspora* isolates revealed spatial separation vertically, but not horizontally, suggesting a spatial niche separation of mycelia (Cano and Bago 2005; Tiwari and Adholeya 2002).

Would such clean and controlled experiments provide information that would help explain natural patterns? Clearly the growth conditions are more uniform (and unrealistic) in terms of nutrient availability, temperature, humidity, host availability, and substrate physical structure. However, the in vitro culture technique is an excellent system allowing estimation of the niche space – fundamental and realised – occupied by different fungi as well as to study interactions, such as competition, herbivory, etc. Thus acquired knowledge could be used to generate a mathematical model which would then need to be validated in complex natural systems.

5.2 Characteristics of AM Fungi Showing Global Distribution

Based on molecular surveys of Glomeromycota, the most widespread fungus appears to be *Glomus intraradices* and closely related taxa such as *G. fasciculatum* and *G. vesiculiferum* (see Öpik et al. 2006b). These fungi have been detected from both disturbed and undisturbed habitats and from many host species (for the latest survey of hosts see Helgason et al. 2007, Table 2). It is possible that the *G. intraradices* group comprises several taxa or at least that the isolates from geographically distant areas are functionally diverse (van der Heijden et al. 2004). *G. intraradices* single spore isolates from a single population have been demonstrated to differentially affect plant growth (Koch et al. 2006). *G. intraradices* isolates of different geographical origin showed variable speed and extent of root colonization as well as effect on plant biomass (Hart and Reader 2002a, 2000b).

Other globally detected root-colonizing Glomeromycota include fungi known both as spores and sequences and fungi known by only one method: *Glomus mosseae*, *G. hoi*, *G.* sp. UY1225 (=MO-G2, Glo3a, GloAb3 by different authors), *G.* sp. Glo10, *G.* sp. MO-G4 (Glo17), *G.* sp. Glo18 (Öpik et al. 2006b). *Glomus mosseae* has been widely used in laboratory studies; it is detected in roots from samples in almost all studied locations (Öpik et al. 2006b), but is not abundant in any. Isolates of this species may show different functional properties (Munkvold et al. 2004). *Glomus hoi* isolates may show functional differentiation and *Glomus* sp. UY 1225 seems to have a broad host range (Helgason et al. 2002). The 'unknown' fungi, though detected from a range of locations and hosts, are also unknown in terms of their functional properties.

5.3 Localized and/or Specialist AM Fungi

Among the root-colonizing AM fungi there are several taxa that appear to inhabit specific ecosystems (Helgason et al. 2007; Öpik et al. 2006b). For example, *Glomus caledonium* and *Glomus* sp. Glo4 have been found from grasslands and arable fields, but not from forests. In contrast, an unidentified sequence type *Glomus* sp. MO-G5 (=Glo2, GloAd3) has been detected from forests and grasslands but not from disturbed (arable or polluted) sites. However, a large proportion of root-colonizing taxa appear to be detected from a single site and/or host species only, including some which are locally abundant (Öpik et al. 2006b). Whether this is a genuine case of rarity or specificity or reflects spatial or temporal patchiness and/or unsaturated sampling, remains to be discovered.

6 Conclusions

There are two options available to make more sense of AM fungal diversity in the field: first, to allocate traits to the observed taxa or, second, to measure the functional diversity in situ. An assumption allowing for meaningfulness of the first is that there is taxonomic variation in the functional traits of interest. Presently, there is supporting evidence for some traits, contradictory for other, and no information for most traits, taxa, and ecosystems. The second option would probably yield the best information on the functions exhibited by fungi in the course of their "normal" ecology and could include information on unculturable fungi; the main question remaining is: how?

We see three main areas where more concerted focus is necessary in order to gain significant progress in understanding natural patterns of Glomeromycota.

1. Characterization of naturally co-occurring Glomeromycota isolates in terms of morphology, development and taxonomy. This would also provide data about variability of traits between isolates of the same species in the same location, or

between locations, once more data are available. In these matters, it is essential to register (and possibly store) the isolates in culture collections and refer to the isolate codes in publications in order that readers can back-trace the source of isolates.

2. Physiological experimentation with (and characterization of) a wider selection and ecologically meaningful sets of Glomeromycota isolates. This would provide us with a more realistic understanding of the functional variability in natural conditions. Ecological meaningfulness might mean isolates/inoculum from one location, isolates/inoculum from the same type of ecosystem or those potentially co-occurring, etc.
3. Description of natural diversity patterns. Both taxonomic and functional patterns of Glomeromycota in nature need to be described. Most importantly, the data need to be comparable and analyzable; thus more attention must be paid to the choice of sampling design, to replication and to the methodological approach, including providing clear details in publications.

References

Abbott LK, Robson AD (1991) Factors influencing the occurrence of vesicular arbuscular mycorrhizas. Agric Ecosyst Environ 35:121–150

Addy HD, Boswell EP, Koide RT (1998) Low temperature acclimation and freezing resistance of extraradical VA mycorrhiza hyphae. Mycol Res 102:582–586

Alexander IJ (2006) Ectomycorrhizas – out of Africa? New Phytol 172:589–591

Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Appl Environ Microbiol 72:4192–4199

Allen M (1996) The ecology of arbuscular mycorrhizas: a look back into the 20th century and a peek into the 21st. Mycol Res 100:769–782

Allen MF (1988) Re-establishment of VA-mycorrhizas following severe disturbance – comparative patch dynamics of a shrub desert and a subalpine volcano. Proc R Soc Edin B 94:63–71

Avio L, Pellegrino E, Bonari E, Giovannetti M (2006) Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. New Phytol 172:347–357

Bago B, Zipfel W, Williams RM, Chamberland H, Lafontaine JG, Webb WW, Piche Y (1998) In vivo studies on the nuclear behaviour of the arbuscular mycorrhizal fungus *Gigaspora rosea* grown under axenic conditions. Protoplasma 203:1–15

Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–958

Bever JD, Schultz PA, Pringle A, Morton JB (2001) Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. BioScience 51:923–934

Bidartondo MI, Redecker D, Hijri I, Wiemken A, Bruns TD, Dominguez L, Sérsic A, Leake JR, Read DJ (2002) Epiparasitic plants specialized on arbuscular mycorrhizal fungi. Nature 419:389–392

Błaszkowski J (1994) Arbuscular fungi and nycorrhizae (Glomales) of the Hel Peninsula, Poland. Mycorrhiza 5:71–88

Boddington CL, Dodd JC (2000) The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. II. Studies in experimental microcosms. Plant Soil 218:145–157

Braunberger PG, Abbott LK, Robson AD (1996) Infectivity of arbuscular mycorrhizal fungi after wetting and drying. New Phytol 134:673–684

Burleigh SH (2001) Relative quantitative RT-PCR to study the expression of plant nutrient transporters in arbuscular mycorrhizas. Plant Sci 160:899–904
Burleigh SH, Cavagnaro T, Jakobsen I (2002) Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J Exp Bot 53: 1593–1601
Cano C, Bago A (2005) Competition and substrate colonization strategies of three polyxenically grown arbuscular mycorrhizal fungi. Mycologia 97:1201–1214
Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265
Clapp JP, Helgason T, Daniell TJ, Young JPW (2002) Genetic studies of the structure and diversity of arbuscular mycorrhizal fungal communities. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 201–224
Dalpé Y, Cranenbrouck S, Seguin S, Declerck S (2005) The monoxenic culture of arbuscular mycorrhizal fungi as a tool for systematics and biodiversity. In: Declerck S, Fortin A, Strullu DG (eds) In vitro vulture of mycorrhizas. Springer, Heidelberg, pp 31–48
Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol Ecol 36:203–209
de la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271
de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012
de Souza FA, Dalpé Y, Declerck S, de la Providencia IE, Séjalon-Delmas N (2005) Life history strategies in Gigasporaceae: insight from monoxenic culture. In: Declerck S, Fortin A, Strullu DG (eds) In vitro culture of mycorrhizas. Springer, Heidelberg, pp 73–91
Dodd JC, Boddington CL, Rodriguez A, Gonzales-Chavez C, Manzur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151
Douds DD, Schenck NC (1991) Germination and hyphal growth of VAM fungi during and after storage in soil ay five matric potentials. Soil Biol Biochem 23:177–183
Drew EA, Murray RS, Smith SE (2006) Functional diversity of external hyphae of AM fungi: ability to colonise new hosts is influenced by fungal species, distance and soil conditions. Appl Soil Ecol 32:350–365
Eissenstat DM, Graham JH, Syvertsen JP, Drouillard DL (1993) Carbon economy of sour orange in relation to mycorrhizal colonization and phosphorus status. Ann Bot 71:1–10
Ellenberg H, Weber HE, Düll R, Wirth V, Werner W, Paulisen D (1991) Zeigerwerte von Pflanzen in Mitteleuropa. Scri Geobot 18:1–248
Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. Proc Natl Acad Sci USA 103:626–631
Franke T, Beenken L, Doring M, Kocyan A, Agerer R (2006) Arbuscular mycorrhizal fungi of the *Glomus*-group A lineage (Glomerales; Glomeromycota) detected in myco-heterotrophic plants from tropical Africa. Mycol Progr 5:24–31
Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575
Green J, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. Trends Ecol Evol 21:501–507
Hart MM, Klironomos JN (2002) Diversity of arbuscular mycorrhizal fungi and ecosystem functioning. In: van der Heijden MGA (ed) Mycorrhizal ecology. Springer, Heidelberg, pp 225–242
Hart MM, Reader RJ (2002a) Host plant benefit from association with arbuscular mycorrhizal fungi: variation due to differences in size of mycelium. Biol Fertil Soils 36:357–366
Hart MM, Reader RJ (2002b) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Heinemeyer A, Ridgway KP, Edwards EJ, Benham DG, Young JPW, Fitter AH (2004) Impact of soil warming and shading on colonization and community structure of arbuscular mycorrhizal fungi in roots of a native grassland community. Global Change Biol 10:52–64

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431

Helgason T, Fitter AH, Young JPW (1999) Molecular diversity of arbuscular mycorrhizal fungi colonising *Hyacinthoides non-scripta* (bluebell) in a seminatural woodland. Mol Ecol 8:659–666

Helgason T, Merryweather JW, Denison J, Wilson P, Young JPW, Fitter AH (2002) Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. J Ecol 90:371–384

Helgason T, Merryweather JW, Young JP, Fitter AH (2007) Specificity and resilience in the arbuscular mycorrhizal fungi of a natural woodland community. J Ecol 95:623–630

Hepper CM, Smith GA (1976) Observations on the germination of *Endogone* spores. Trans Br Mycol Soc 66:189–194

Hepper CM, Azcon-Aguilar C, Rosendahl S (1988) Competition between three species of *Glomus* used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol 110:207–215

Hijri I, Sykorova Z, Oehl F, Ineichen K, Mäder P, Wiemken A, Redecker D (2006) Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol Ecol 15:2277–2289

Husband R, Herre EA, Turner SL, Gallery R, Young JPW (2002a) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Mol Ecol 11:2669–2678

Husband R, Herre EA, Young JPW (2002b) Temporal variation in the arbuscular mycorrhizal communities colonising seedlings in a tropical forest. FEMS Microbiol Ecol 42: 131–136

Jacquot-Plumey E, van Tuinen D, Chatagnier O, Gianinazzi S, Gianinazzi-Pearson V (2001) 25S rDNA-based molecular monitoring of glomalean fungi in sewage sludge-treated field plots. Environ Microbiol 3:525–531

Jakobsen I, Abbott LK, Robson AD (1992a) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1: Spread of hyphae and phosphorus inflow into roots. New Phytol 120:370–380

Jakobsen I, Abbott LK, Robson AD (1992b) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 2: Hyphal transport of 32P over defined distances. New Phytol 120:509–516

Jansa J, Mozafar A, Frossard E (2005) Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176

Jasper DA, Abbott LK, Robson AD (1991) The effect of soil disturbance on vesicular-arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytol 118:471–476

Jasper DA, Abbott LK, Robson AD (1993) The survival of infective hyphae of vesicular-arbuscular fungi in dry soil: an interaction with sporulation. New Phytol 124:473–479

Johnson NC, Tilman D, Wedin D (1992) Plant and soil controls on mycorrhizal fungal communities. Ecology 73:2034–2042

Juge C, Samson J, Bastien C, Vierheilig H, Coughlan A, Piché, Y (2002) Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period. Mycorrhiza 12:37–42

Jumpponen A, Trowbridge J, Mandyam K, Johnson L (2005) Nitrogen enrichment causes minimal changes in arbuscular mycorrhizal colonization but shifts community composition evidence from rDNA data. Biol Fertil Soils 41:217–224

Kabir Z, O'Halloran IP, Hamel C (1997) Overwinter survival of arbuscular mycorrhizal hyphae is favored by attachment to roots but diminished by disturbance. Mycorrhiza 7:197–200

Kiers ET, van der Heijden MGA (2006) Mutualistic stability in the arbuscular mycorrhizal symbiosis: exploring the hypotheses of evolutionary cooperation. Ecology 87:1627–1636

Kjøller R, Rosendahl S (2000) Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226:189–196
Kjøller R, Rosendahl S (2001) Molecular diversity of glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. Mycol Res 105:1027–1032
Klimes L, Klimesova J (1999) CLO-PLA2 – a database of clonal plants in central Europe. Plant Ecol 141:9–19
Klironomos JN (2003) Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84:2292–2301
Klironomos JN, Rillig MC, Allen MF (1999) Designing belowground field experiments with the help of semi-variance and power analyses. Appl Soil Ecol 12:227–238
Klironomos JN, Hart MM, Gurney JE, Moutoglis P (2001) Interspecific differences in the tolerance of arbuscular mycorrhizal fungi to freezing and drying. Can J Bot 79:1161–1166
Koch AM, Croll D, Sanders IR (2006) Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. Ecol Lett 9:103–110
Lekberg Y, Koide RT, Rohr JR, Drich-Wolfe L, Morton JB (2007) Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. J Ecol 95:95–105
Lilleskov EA, Parrent JL (2007) Can we develop general predictive models of mycorrhizal fungal commuity-environment relationships? New Phytol 174:250–256
Maherali H, Klironomos JN (2007) Influence of phylogeny on fungal community assembly and ecosystem functioning. Science 316:1746–1748
Mangan SA, Adler GH (2002) Seasonal dispersal of arbuscular mycorrhizal fungi by spiny rats in a neotropical forest. Oecologia 131:587–591
Mathimaran N, Ruh R, Jama B, Verchot L, Frossard E, Jansa J (2007) Impact of agricultural management on arbuscular mycorrhizal fungal communities in Kenyan ferralsol. Agric Ecosyst Environ 119:22–32
McGee PA (1989) Variation of propagule numbers of vesicular-arbuscular mycorrhizal fungi in a semi-arid soil. Mycol Res 92:28–33
McGee PA, Pattinson GS, Heath RA, Newman CA, Allen SJ (1997) Survival of propagules of arbuscular mycorrhizal fungi in soils in eastern Australia used to grow cotton. New Phytol 135:773–780
McGill BJ, Enquist BJ, Weiher E, Westoby M (2006) Rebuilding community ecology from functional traits. Trends Ecol Evol 21:178–185
Merryweather J, Fitter A (1998a) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta*. I. Diversity of fungal taxa. New Phytol 138:117–129
Merryweather J, Fitter A (1998b) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* II. Seasonal and spatial patterns of fungal populations. New Phytol 138:131–142
Miller RM, Reinhardt DR, Jastrow JD (1995) External hyphal production of vesicular-arbuscular mycorrhizal fungi in pasture and tallgrass prairie communities. Oecologia 103:17–23
Molina R, Massicotte HB, Trappe JM (1992) Specificity phenomena in mycorrhizal symbioses: community-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, New York, pp 357–423
Mummey DL, Rillig MC (2006) The invasive plant species *Centaurea maculosa* alters arbuscular mycorrhizal fungal communities in the field. Plant Soil 288:81–90
Mummey DL, Rillig MC, Holben WE (2005) Neighboring plant influences on arbuscular mycorrhizal fungal community composition as assessed by T-RFLP analysis. Plant Soil 271:83–90
Munkvold L, Kjøller R, Vestberg M, Rosendahl S, Jakobsen I (2004) High functional diversity within species of arbuscular mycorrhizal fungi. New Phytol 164:357–364
Nielsen KB, Kjøller R, Olsson PA, Schweiger PF, Andersen FO, Rosendahl S (2004) Colonisation and molecular diversity of arbuscular mycorrhizal fungi in the aquatic plants *Littorella uniflora* and *Lobelia dortmanna* in southern Sweden. Mycol Res 108:616–625
O'Neill EG, O'Neill RV, Norby RJ (1991) Hierarchy theory as a guide to mycorrhizal research on large-scale problems. Environ Poll 73:271–284
Olsson PA, Wilhelmsson P (2000) The growth of external AM fungal mycelium in sand dunes and in experimental systems. Plant Soil 226:161–169

Olsson PA, Jakobsen I, Wallander H (2002) Foraging and resource allocation strategies of mycorrhizal fungi in a patchy environment. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology, vol 157. Springer, Heidelberg, pp 93–115

Öpik M, Moora M, Liira J, Kõljalg U, Zobel M, Sen R (2003) Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. New Phytol 160:581–593

Öpik M, Moora M, Liira J, Rosendahl S, Zobel M (2006a) Comparison of communities of arbuscular mycorrhizal fungi in roots of two *Viola* species. Proc Est Acad Sci Biol Ecol 55:3–14

Öpik M, Moora M, Liira J, Zobel M (2006b) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J Ecol 778:778–790

Pärtel M, Zobel M (2007) Dispersal limitation may result in the unimodal productivity-diversity relationship: a new explanation for a general pattern. J Ecol 95:90–94

Pearson JN, Jakobsen I (1993) Symbiotic exchange of carbon and phosphorus between cucumber and three arbuscular mycorrhizal fungi. New Phytol 124:481–488

Pearson JN, Abbott LK, Jasper DA (1993) Mediation of competition between two colonizing VA mycorrhizal fungi by the host plant. New Phytol 123:93–98

Raab PA, Brennwald A, Redecker D (2005) Mitochondrial large ribosomal subunit sequences are homogeneous within isolates of *Glomus* (arbuscular mycorrhizal fungi, Glomeromycota). Mycol Res 109:1315–1322

Ramette A, Tiedje J (2007) Biogeography: an emerging cornerstone for understanding prokaryotic diversity, ecology, and evolution. Micr Ecol 53:197–207

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Redecker D, Raab P (2006) Phylogeny of the Glomeromycota (arbuscular mycorrhizal fungi): recent developments and new gene markers. Mycologia 98:885–895

Redecker D, Hijri I, Wiemken A (2003) Molecular identification of arbuscular mycorrhizal fungi in roots: perspectives and problems. Folia Geobot 38:113–124

Russell J, Bulman S (2005) The liverwort *Marchantia foliacea* forms a specialized symbiosis with arbuscular mycorrhizal fungi in the genus *Glomus*. New Phytol 165:567–579

Safir GR, Coley SC, Siqueira JO, Carlson PS (1990) Improvement and synchronization of VA mycorrhiza fungal spore germination by short-term cold storage. Soil Biol Biochem 22:109–111

Saito K, Suyama Y, Sato S, Sugawara K (2004) Defoliation effects on the community structure of arbuscular mycorrhizal fungi based on 18S rDNA sequences. Mycorrhiza 14:363–373

Sanders IR (2004) Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? New Phytol 164:415–418

Santos JC, Finlay RD, Tehler A (2006) Molecular analysis of arbuscular mycorrhizal fungi colonising a semi-natural grassland along a fertilisation gradient. New Phytol 172:159–168

Scheublin TR, Ridgway KP, Young JPW, van der Heijden MGA (2004) Nonlegumes, legumes, and root nodules harbour different arbuscular mycorrhizal fungal communities. Appl Environ Microbiol 70:6240–6246

Scheublin TR, van der Heijden MGA (2006) Arbuscular mycorrhizal fungi colonize nonfixing root nodules of several legume species. New Phytol 172:732–738

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Simon L, Lalonde M, Bruns TD (1992) Specific amplification of 18S fungal ribosomal genes from VA endomycorrhizal fungi colonizing roots. Appl Environ Microbiol 58:291–295

Smith FA, Jakobsen I, Smith SE (2000) Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. New Phytol 147:357–366

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, London

Stutz JC, Morton JB (1996) Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. Can J Bot 74:1883–1889

Suzuki J-I, Herben T, Maki M (2004) An under-appreciated difficulty: sampling plant populations for analysis using molecular markers. Evol Ecol 18:625–646

Tadych M, Błaszkowski J (2000) Arbuscular fungi and mycorrhizae (Glomales) of the Sloviçski National Park, Poland. Mycotaxon 74:463–482

Tiwari P, Adholeya A (2002) In vitro co-culture of two AMF isolates *Gigaspora margarita* and *Glomus intraradices* on Ri T-DNA transformed roots. FEMS Microbiol Lett 206:39–43

Tommerup IC (1985) Inhibition of spore germination of vesicular-arbuscular mycorrhizal fungi in soil. Trans Br Mycol Soc 85:367–278

Treseder KK, Cross A (2006) Global distributions of arbuscular mycorrhizal fungi. Ecosystems 9:305–316

Turnau K, Ryszka P, Gianinazzi-Pearson V, Tuinen Dv (2001) Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 10:169–174

Vallino M, Massa N, Lumini E, Bianciotto V, Berta G, Bonfante P (2006) Assessment of arbuscular mycorrhizal fungal diversity in roots of *Solidago gigantea* growing in a polluted soil in Northern Italy. Environ Microbiol 8:971–983

van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

van der Heijden MGA, Sanders IR (2002) Mycorrhizal ecology. Springer, Heidelberg

van der Heijden MGA, Scheublin TR (2007) Functional traits in mycorrhizal ecology: their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning. New Phytol 174:274–250

van der Heijden MGA, Scheublin TR, Brader A (2004) Taxonomic and functional diversity in arbuscular mycorrhizal fungi – is there any relationship? New Phytol 164:201–204

van der Heijden MGA, Streitwolf–Engel R, Riedl R, Siegrist S, Neudecker A, Ineichen K, Boller T, Wiemken A, Sanders IR (2006) The mycorrhizal contribution to plant productivity, plant nutrition and soil structure in experimental grassland. New Phytol 172:739–752

Vandenkoornhuyse P, Husband R, Daniell TJ, Watson IJ, Duck JM, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Mol Ecol 11:1555–1564

Walker C, Trappe JM (1993) Names and epithets in the Glomales and Endogonales. Mycol Res 97:339–344

Warner NJ, Allen MF, MacMahon JA (1987) Dispersal agents of vesicular-arbuscular mycorrhizal fungi in a disturbed arid ecosystem. Mycologia 79:721–730

Wardle DA (1992) A comparative assessment of factors which influence microbial biomass carbon and nitrogen levels in soil. Biol Rev Camb Philos Soc 67:321–358.

Wirsel SGR (2004) Homogenous stands of a wetland grass harbour diverse consortia of arbuscular mycorrhizal fungi. FEMS Microbiol Ecol 48:129–138

Wolfe BE, Mummey DL, Rillig MC, Klironomos JN (2007) Small-scale spatial heterogeneity of arbuscular mycorrhizal fungal abundance and community composition in a wetland plant community. Mycorrhiza 17:175–183

Wubet T, Weiss M, Kottke I, Teketay D, Oberwinkler F (2006) Phylogenetic analysis of nuclear small subunit rDNA sequences suggests that the endangered African Pencil Cedar, *Juniperus procera*, is associated with distinct members of *Glomeraceae*. Mycol Res 110:1059–1069

Wubet T, Weiss M, Kottke I, Teketay D, Oberwinkler F (2004) Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. New Phytol 161:517–528

Yamato M, Iwase K (2005) Community analysis of arbuscular mycorrhizal fungi in a warm-temperate deciduous broad-leaved forest and introduction of the fungal community into the seedlings of indigenous woody plants. Mycoscience 46:334–342

Mycorrhiza Helper Bacteria

Mika T. Tarkka(✉), Pascale Frey-Klett

1 Introduction

Mycorrhizal symbiosis should not be considered merely as a bipartite plant–fungus interaction, but should instead incorporate the associated organisms. These mycorrhiza-associated organisms are known to influence each other mutually, the outcome of which is described as the "mycorrhizosphere" (Foster and Marks 1966; Meyer and Linderman 1986; Frey-Klett and Garbaye 2005). The mycorrhizosphere comprises mycorrhizas, extramatrical mycelium and the associated microorganisms. In the same way the rhizospheres exert a pressure on microbial populations (Barea et al. 2005), the mycorrhizal roots and hyphae of mycorrhizal fungi (MF) shape the bacterial species composition due to root and hyphal exudation and turnover (Bowen 1993; Morgan et al. 2005). This "mycorrhizosphere effect" may lead to improved plant nutrition, growth and disease resistance (Linderman 1988; Frey-Klett et al. 2005). Determining the functional significance of the mycorrhizosphere organisms for plant productivity presents a major challenge for the future (Artursson et al. 2006).

The presence of bacteria that are directly involved in mycorrhiza formation was first indicated by the studies of Bowen and Theodorou (1979) which showed that some bacterial isolates promoted and others inhibited the colonization of *Pinus radiata* roots by *Rhizopogon luteolus*. In subsequent work the presence of bacteria able to promote mycorrhiza formation was confirmed in ectomycorrhiza (ECM) (Garbaye and Bowen 1987; de Oliveira and Garbaye 1989), in arbuscular mycorrhiza (AM) (Meyer and Linderman 1986; Ames 1989) and suggested in orchid mycorrhizal associations (Wilkinson et al. 1989). The bacteria able to promote mycorrhizal development were then collectively named as MHB (mycorrhiza helper bacteria; Duponnois and Garbaye 1991; Garbaye 1994). Their presence in other types of mycorrhizal associations may be expected, but has not been investigated thus far.

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In this review, we will briefly cover the origin and taxonomy of the MHB. An emphasis will be given for the thus far characterized mechanisms that lead to enhanced development of mycorrhizal symbiosis, and on the specificity of this tripartite interaction, which is reflected by the promotion of some and inhibition of other fungal species by individual MHB isolates. Finally, a short overview will be given on the possible applications for MHB in forestry and agriculture. For a deeper insight into bacterium–fungus interactions in mycorrhizas, mycorrhiza–PGPR interactions and other beneficial interactions occurring in the mycorrhizosphere, the reader is advised to take notice of recent reviews (Barea et al. 2005; Morgan et al. 2005; Artursson et al. 2006; Marschner and Timonen 2006; Reddy and Satyanarayana 2006; Frey-Klett et al. 2007; Gamalero et al., this volume), and, for a broader coverage of early literature or ecological aspects regarding the MHB, on the reviews by Garbaye (1991, 1994) and Duponnois (2006).

2 Helper Strains: Origin and Taxonomy

The presence of MHB as an ubiquitous group of micro-organisms and important for mycorrhizal symbiosis is suggested by the following findings: (1) MHB have been found whenever they have been looked for, (2) they are present in very different habitats, (3) many of these bacteria seem to be closely associated with MF, and (4) MHB can be found from taxonomically diverse bacterial groups.

MHB have been isolated from very different habitats. Apart from mycorrhizas and from the mycorrhizospheres, these bacteria have been isolated from ECMF fruiting bodies, AMF spores, galls, termite mounds, and heavy metal-contaminated soils (Garbaye and Bowen 1989; Founoune et al. 2002; Xavier and Germida 2003; Gamalero et al. 2003; Duponnois et al. 2006; Vivas et al. 2003d). Because endocellular bacteria have been reported for a long time in different AM fungi (Bianciotto et al. 1996; Bianciotto and Bonfante 2002) and more recently also in the ectomycorrhizal basidiomycete *Laccaria bicolor* and the ascomycete *Tuber borchii* (Barbieri et al. 2000; Bertaux et al. 2003), one should wonder if these bacteria can harbor mycorrhiza helper activity. Preliminary results have shown that the *Paenibacillus* strain which was suspected to intracellularly colonize *L. bicolor* had a promoting effect on the Douglas fir–*L. bicolor* symbiosis (Frey-Klett, unpublished).

The majority of bacteria from the ectomycorrhizal mantle analyzed by Garbaye and Bowen (1989) had a stimulating effect on the mycelial growth of *R. luteolus* and mycorrhiza formation. Ames (1989) tested 12 actinomycete isolates from AM fungal spores on mycorrhiza formation in onion seedlings, and observed that 7 of these isolates were able to stimulate AM establishment. This indicates that not only single species but microbial communities may have evolved to live in close association with mycorrhizal fungi. Same conclusion was also indicated by the analyses of Frey-Klett et al. (2005), where the authors reported that ectomycorrhizospheres of Douglas fir select for plant-beneficial pseudomonads, while reducing strains that were inhibitory to ECM development.

Fluorescent pseudomonads and bacilli have been frequently reported as MHB of ECM symbiosis (Garbaye and Bowen 1989; Garbaye and Duponnois 1992; Founoune et al. 2002; Frey-Klett et al. 2005), but the MHB also include bacterial species from the genera *Burkholderia, Rhodococcus,* and *Streptomyces* (Poole et al. 2001; Schrey et al. 2005). The MHB of AM associations include actinomycetes (Ames 1989; Abdel-Fattah and Mohamedin 2000), pseudomonads (Gryndler and Vosatka 1996; Gamalero et al. 2004), and members of the genus *Alcaligenes* (Will and Sylvia 1990) *Acetobacter* (Paula et al. 1992), *Azospirillum* (Rao et al. 1985), *Bacillus* (Vivas et al. 2003a), *Enterobacter* (Toro et al. 1997), *Klebsiella* (Will and Sylvia 1990; Paula et al. 1992), *Bradyrhizobium* and *Rhizobium* (Xie et al. 1995; Requena et al. 1997). Fluorescent pseudomonads stimulated symbiotic germination of the orchid *Pterostylis vittata* (Wilkinson et al. 1989), suggesting that bacteria may also improve orchid symbiosis.

It is obvious that MHB should be readily cultivable to facilitate their use in controlled mycorrhization assays. However, cultivation-based approaches lead to the isolation of only small proportions of the bacterial species that exist in nature (Torsvik et al. 1990). Mogge et al. (2000) argued that this would suggest that cultivable MHB very likely represent only a small proportion of total mycorrhizosphere bacteria. However, a similarly reasonable suggestion would be that the MHB represent a dominant group of bacteria, an easily cultivable subset of which has been characterized thus far.

3 The Helper Mechanisms

The extent of mycorrhizal colonization depends on the interactions between abiotic and biotic environmental parameters, fungal physiology, and root susceptibility to infection. MHB may promote the mycorrhizal infection rate at different stages of the bacterium–fungus–plant interaction. For instance, pre-infection phases such as spore germination and mycelial growth through soil and on the root surface may be enhanced by MHB, and the root susceptibility to infection may be increased (Bowen 1993). The observation that a similar MHB response could be observed in simple aseptic culture systems as well as in greenhouse experiments (reviewed in Garbaye 1994) has enabled the use of axenic experimental models to address the mechanisms involved in the enhancement of mycorrhiza development. Five major hypotheses explaining the helper effect were presented by Garbaye (1994) (Fig. 1), and some evidence has been presented for each of these putative mechanisms. (For a recent review, refer to Frey-Klett et al. 2007).

3.1 Promoted Germination of Fungal Propagules

The exudates of MHB often stimulate fungal spore germination. Mosse (1962) showed that some rhizosphere bacteria and their culture filtrates were able to stimulate *Glomus mosseae* spore germination; these observations were confirmed

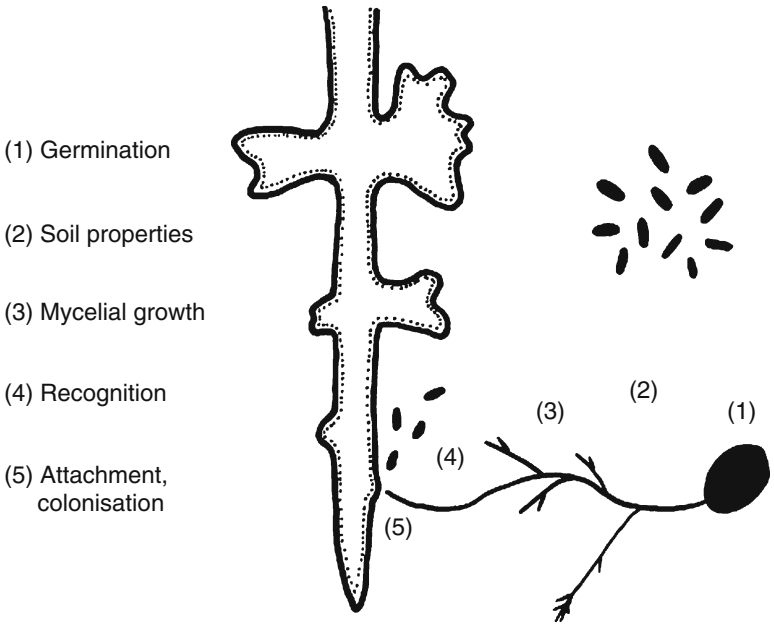


Fig. 1 The sites of action for mycorrhiza helper bacteria. Adapted from Garbaye (1994)

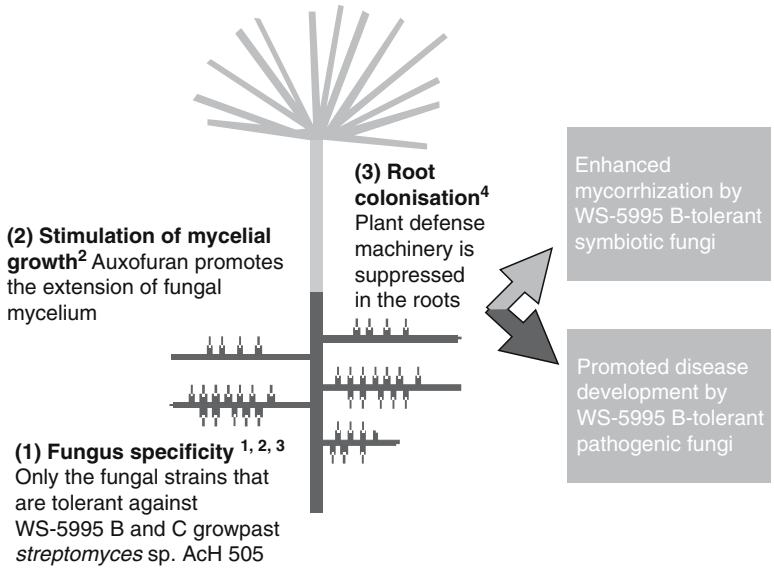
with *G. mosseae* and *G. versiforme* (Mayo et al. 1986; Azcón-Aguilar et al. 1986). The inoculation of sea oats (*Unicola paniculata*) roots with *Klebsiella pneumoniae* led to increased spore germination and faster extension of *G. deserticola* hyphae (Will and Sylvia 1990). Mycorrhiza formation in pot cultures was also increased following bacterial inoculation (Will and Sylvia 1990). Xavier and Germida (2003) observed that a substantial fraction of bacteria from AMF spore cell walls were able to promote *G. clarum* spore germination when a direct contact between the spores and bacteria existed, whereas some bacterial isolates were inhibiting spore germination by producing antagonistic volatiles. Actinomycetes have been observed on the surface of AM fungal spores, and, depending on the species, they either promoted or suppressed AM spore germination. Whereas Krishna et al. (1982) reported an antagonism between a *Streptomyces* sp. and *G. fasciculatus*, Mugnier and Mosse (1987) observed enhanced spore germination rates using a different *Streptomyces* sp. and *G. mosseae*. ECM fungal spore germination has received surprisingly little attention with respect to helper bacteria, whereas it is well known that, depending on the ECM fungi, the bacterial communities inhabiting the sporocarp can be very numerous and diverse (e.g., *Cantharellus:* Danell et al. 1993; *Tuber*: Bedini et al. 1999; Gazzanelli et al. 1999; Barbieri et al. 2005). Fries (1987) reported that basidiospore germination is stimulated by both soil yeasts and bacteria, and Ali and Jackson (1989) showed that *Corynebacterium* and several *Pseudomonas* isolates were able to stimulate basidiospore germination.

3.2 Promoted Mycelial Growth

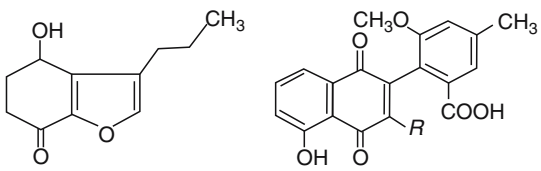
Fungus–bacterium co-cultures are easily implemented and thus were often used as first indicators for the screening of MHB strains promoting hyphal growth. If MHB inoculation leads to increased mycelial biomass in the soil, the occurrence of root–fungus encounters should increase too, resulting in faster mycorrhization (Brulé et al. 2001). In line with this hypothesis, a significant correlation has been shown to exist between improved mycelial extension and promoted mycorrhiza establishment (Garbaye and Bowen 1989; Garbaye and Duponnois 1992; Gryndler and Vosatka 1996; Founoune et al. 2002; Schrey et al. 2005; Riedlinger et al. 2006). For the ECM fungus *Amanita muscaria*, however, Maier (2003) observed that the fungal mycelial density decreases in co-culture with the MHB *Streptomyces* sp. AcH 505. This suggested that the MHB effect of AcH 505 is caused by faster spread of the mycelial front but not by higher mycelial density.

The relative ease to perform bacterium–fungus co-cultures in a reproducible manner has enabled Deveau et al. (2007) and Schrey et al. (2005) to perform an analysis on ectomycorrhizal fungal gene expression levels during the interaction with helper bacterial strains. Using a microarray approach, Deveau et al. (2007) identified early stage-responsive genes presumably involved in the priming effect of the helper bacterial strain *P. fluorescens* BBc6R8 on the growth and morphology of its ectomycorrhizal fungal associate *L. bicolor* A238N. In the case of the interaction between *Streptomyces* sp. AcH 505 and the ECM fungus *A. muscaria*. Schrey et al. (2005) and Tarkka et al. (2006) demonstrated that the fungal genes upregulated in co-culture with AcH 505 included members of signal transduction pathways and genes related to cell stress and cell growth, metabolism and cell structure. One of the analyzed genes, the cyclophilin gene *AmCyp40*, was similarly upregulated by the cell-free culture supernatants of AcH 505 and *Streptomyces setonii* AcH 1003, but not by those of *S. argenteolus* AcH 504. Since AcH 505 and AcH 1003 promote the growth of *A. muscaria* and enhance mycorrhization, but AcH 504 does not, this suggested that *AmCyp40* could respond to MHB in general (Schrey et al. 2005). The fact that *AmCyp40* responds to cell stress in general (S. Schrey, unpublished) indicates that AcH 505 and AcH 1003 produce stress-inducing substances, later confirmed with AcH 505 (Riedlinger et al. 2006). AcH 505 also posses an effect on hyphal architecture, since the *A. muscaria*-AcH 505 dual culture hyphae are thinner than the non-inoculated hyphae (Maier 2003).

To screen for bacterial compounds responsible for growth promotion by *Streptomyces* sp. AcH 505, the suspension cultures of the bacterium were analyzed for dominant secondary metabolites. Several chromatographic steps were used to isolate a fungal growth-promoting factor in pure form, and its structure was elucidated by nuclear magnetic resonance spectroscopy (Riedlinger et al. 2006; Keller et al., unpublished). The fungal growth promoter was a novel compound, classified due to its auxin related structure as auxofuran (Fig. 2b). The production rate of auxofuran into the culture medium ranged from 10 nM to 10 µM, ideal for growth promotion of fungi, since all tested homobasidiomycete fungi respond to 1 nM to 1 µM auxofuran (Riedlinger et al. 2006; M. Tarkka, unpublished). In the presence of *A. muscaria,* AcH 505 produces 4-fold more auxofuran than in single culture, due to the acidification of the culture


(3) Root colonisation[4] Plant defense machinery is suppressed in the roots
Enhanced mycorrhization by WS-5995 B-tolerant symbiotic fungi
(2) Stimulation of mycelial growth[2] Auxofuran promotes the extension of fungal mycelium
Promoted disease development by WS-5995 B-tolerant pathogenic fungi
(1) Fungus specificity [1, 2, 3] Only the fungal strains that are tolerant against WS-5995 B and C growpast *streptomyces* sp. AcH 505

a



Auxofuran

WS-5995 B: *R* = H

WS-5995 C: *R* = OH

b

Fig. 2 The secondary metabolites produced by *Streptomyces* sp. AcH 505 and the MHB effect. **a** Model explaining the interaction between AcH 505, fungi and plants. References: [1]Maier et al. (2004); [2]Schrey et al. (2005); [3]Riedlinger et al. (2006); [4]Lehr et al.(upublished). **b** Dominant secondary metabolites of AcH 505, auxofuran, WS-5995 B and WS-5995 C

medium by fungal exudates. The expression levels of *A. muscaria* gene acetoacyl co A synthetase (*AmAacs*) were upregulated by auxofuran treatment, indicating an activation of sterol biosynthesis by this substance (Riedlinger et al. 2006).

The fungal influence on the production of a bacterial growth-promoting factor was also indirectly suggested in a recent study by Duponnois and Kisa (2006). These authors showed that the MHB *Pseudomonas monteilii* produces currently unknown gaseous compounds that increase the growth rate of *Pisolithus albu,* when the fungus is grown on tryptic soy broth agar or on a minimal medium with trehalose, a carbohydrate that it is frequently accumulated in fungal mycelium. The stimulatory volatiles are not produced when the bacteria are grown on a minimal medium with simple organic acids, chitin, or starch as carbohydrate sources. With *Streptomyces*

sp. AcH 505, we have observed that the fungal growth stimulator auxofuran is not produced at pH values lower than 5 or higher than 7.5 (Riedlinger 2006). As the organic acids lower and chitin increases the pH significantly, one should expect that medium pH might also have an influence on stimulatory volatile production by *P. monteilii*. The volatiles produced by MHB may also have growth inhibitory effects against some fungal species. With the Douglas fir–*L. laccata* system, Garbaye and Duponnois (1992) observed that at least some of the growth inhibitory factors produced by the MHB were volatiles. The nature of these compounds is still unknown, but could perhaps be unraveled by gas chromatography/mass spectrometry.

AM infection rate of the roots with *Glomus fistulosum* and the growth rate of soil substrate hyphae were significantly higher when the fungus was co-inoculated with *Pseudomonas putida* or with the culture supernatant of the bacterium (Gryndler and Vosatka 1996). The application of a low molecular weight fraction from the *P. putida* culture increased mycorrhiza formation and the extension of extraradical hyphae (Vosatka and Gryndler 1999), indicating that the effective substances were in this fraction. Although not yet tested for mycorrhization, the investigations on *Paenibacillus validus–Glomus intraradices* interaction have already given insight into the mechanisms that enhance AM mycelial development. Hildebrandt et al. (2002) showed that this otherwise obligately symbiotic fungus could grow and sporulate in fungus–bacterium co-cultures. A specific carbon source, raffinose, was detected in bacterial cultures, and mycelial growth was apparently supported by this sugar (Hildebrandt et al. 2006). The production of fertile spores did not take place after raffinose applications, indicating that in addition to raffinose other bioactive substances are involved in the *Paenibacillus validus–G. intraradices* interaction.

3.3 Modification of the Mycorrhizosphere Soil

In a long-term survey, Brulé et al. (2001) trapped fungal mycelium with Douglas fir seedlings, with and without the influence of *P. fluorescens* BBc6. The authors suggested that BBc6 promotes the survival of the fungal inoculum in the soil, since they observed a significant positive bacterial influence on fungal biomass only after autoclaving the nursery soil prior to adding bacteria and fungal inoculum, e.g., under adverse conditions for fungal development (Brulé et al. 2001). The data from Brulé et al. (2001) suggest that, with certain fungus–plant–substrate combinations, the MHB effect may only be observable when fungal growth is inhibited. Many of the soil microbes, including mycorrhizal fungi, produce toxic metabolites to suppress the growth of competitors. Duponnois and Garbaye (1990) analyzed how the MHB influenced the concentrations of antagonistic substances produced by mycorrhizal fungi. They could show that the bacteria were able to detoxify the liquid media from the inhibitory fungal metabolites. Helper bacteria could perhaps also suppress the production of toxic substances by soil microbes. We have found that antibiotic production by *Streptomyces* sp. AcH 505 can be suppressed by acidic substance production by *Amanita muscaria* (Riedlinger et al. 2006). The

presence of organic acid-producing bacteria in the soil suggests that some MHB may possess a similar activity.

Environmental parameters, e.g., drought or pollution stress, show a strong influence on mycorrhizal symbiosis and on the extent of mycorrhiza helper effect. By subjecting plants to polyethylene glycol- (PEG) induced drought stress conditions, Vivas et al. (2003a) addressed the question if the influence of a *Bacillus* sp. on the colonization or on physiological activities of arbuscular mycorrhizal fungi depends on the water supply. Bacterial inoculation with *Bacillus* sp. had a stronger positive influence on the colonization intensity and arbuscule abundance in the mycorrhizal roots when the plants were subjected to drought stress. Moreover, succinate dehydrogenase staining, indicative of active intraradical AMF mycelium, was also much stronger in the co-inoculated lettuce roots subjected to drought. In experiments using Cd- or Zn-contaminated substrates, the AM colonization and the development of extraradical mycelium in plants colonized by *G. mosseae* was observed to increase in the presence of *Brevibacillus brevis* (Vivas et al. 2003b, 2003c). These effects could be related to an increased carbohydrate transport from the host plant to the fungus. In a subsequent study, Vivas et al. (2005) were able to show that the bacteria had a strong positive impact on spore germination and on presymbiotic fungal growth in heavy metal-contaminated solutions. Bacterial inoculation not only reduced damage to *G. mossae* hyphae but even resulted in increased hyphal growth from 195% (without Cd) to 254% (with Cd solution). The effect was similarly strong under Zn treatment where mycelial growth ranged from 125% (without Zn) to 232% (with Zn solution).

3.4 Host Recognition and Changes in Root System Architecture

The recognition process between the host plant and the mycorrhizal fungus includes the reception of plant signals by the fungal mycelium, chemotrophic hyphal extension growth to the prospective infection site, and characteristic changes in mycelial and hyphal morphology. Xie et al. (1995) showed that MHB may be able to enhance the production of stimulatory signals that direct mycelial growth towards the root. *Bradyrhizobium japonicum* stimulated AM colonization by inducing changes in the host plant's flavonoid spectrum. Mycorrhization of soybeans was not only enhanced in the presence of Nod factor-producing rhizobia but also by exogenous application of specific Nod factors and flavonoids, suggesting that the Nod factor-induced stimulation of mycorrhizal colonization in soybean roots is mediated by plant flavonoids. As specific flavonoids produced by the roots of host plants also serve as signaling molecules promoting ECM fungi (Lagrange et al. 2001), the helper mechanism involving modulation of plant–fungus signaling should be further investigated, as has not been the case so far.

Lateral root production can be positively influenced by MHB (Garbaye 1994; Poole et al. 2001; Vivas et al. 2003d; Schrey et al. 2005), probably due to the production of auxins or auxin-related substances by the bacteria. The formation of novel root tips may lead to the establishment of more mycorrhizas, as the density of colonization sites per soil volume increases. However, the mycorrhizal rate (mycorrhizas / total

fine roots) may decrease if the lateral roots form in areas poor in fungal hyphae. In some cases, MHB exhibited differential effects on the development of the root system. The *Bacillus* strain isolated by Bending et al. (2002) increased the formation only of first order ECM roots, but *Burkholderia* and *Rhodococcus* strains isolated by Poole et al. (2001) increased the formation only of secondary order ECM roots in Scots pine. Localization of the bacteria did not reveal how the bacteria induced these specific root branching patterns, and the authors suggested that the bacteria possessed differential hormonal effects on the Scots pine roots (Poole et al. 2001). During the development of ECM in pine short roots, dichotomous (root tip) branching leads to the formation of coralloid mycorrhizal roots, producing as many as 40 root tips at their maturity. *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67, two strains isolated from *L. rufus* mycorrhiza, were found to promote dichotomous root branching in Scots pine (*Pinus sylvestris*) seedlings. Aspray et al. (2006a) suggested that the number of individual root tips rather than absolute number of mycorrhizal roots may be an important previously overlooked parameter for defining MHB effects.

Hormonal effects may be responsible for the MHB effect exhibited by some bacteria towards symbiotic germination of orchids (Wilkinson et al. 1989, 1994). From seven tested bacterial strains isolated from inside the underground parts of the orchid *P. vittata*, three were able to significantly promote symbiotic seed germination, one showed no difference to the uninoculated control, and three significantly suppressed seedling development. Bacterial auxin production may be a key factor behind enhanced symbiotic germination, as auxin treatments also enhanced symbiotic germination (Wilkinson et al. 1994).

The morphology of fungal mycelia upon mycorrhization in the presence of MHB has not received much attention. We have observed thinning of the hyphae of the ECM fungus *Amanita muscaria* and changed cytoskeletal architecture owing to the influence of *Streptomyces* sp. AcH 505. These changes in hyphal cell structure can be induced by the application of cell-free AcH 505 culture filtrates into the culture medium (S. Schrey et al., unpublished). Similarly, in in vitro co-cultures of *L. bicolor* S238N with MHB bacterial strains, Deveau et al. (2007) observed significant morphological modifications of the hyphal apex density and branching angles, which depended on the bacterial strains.

3.5 Receptivity of the Roots

According to the fifth hypothesis, the bacterium facilitates the colonization of the root system while growing in the rhizosphere prior to the contact between the mycorrhizal fungus and the host plant. This could occur through controlled production by the MHB of cell wall digesting enzymes, permitting the enhanced penetration of the roots by the fungal hyphae and easing their spread inside the root tissues. The suppression of plant defense response prior to fungal colonization could also potentially lead to enhanced mycorrhization.

The recent work of Aspray et al. (2006b) showed, that *Paenibacillus* sp. EJP73 only promoted mycorrhiza establishment in Scots pine when the bacterium was in direct contact with the short roots. The application of EJP73 culture filtrate showed

no positive effect on mycorrhiza development, suggesting that three non-exclusive hypotheses: (1) the bacterium exudes the effector molecules only when in contact with the roots, (2) the effectors are attached to the bacterial cell wall, and/or (3) the substances are short-lived and produced by live bacteria. Softening of root cell walls by the bacteria could also render the plants more susceptible to fungal colonization. The early work by Mosse (1962) showed that some microorganisms belonging to the genus *Pseudomonas* produce cell wall degrading enzymes and promote the establishment of AM on clover roots. Bacterial culture filtrates and enzyme preparations were similarly efficient in promoting AM development, an indication of a role for these enzymes in the MHB effect.

According to the current model for mycorrhizal symbiosis, mycorrhizal fungi evoke a temporary defense response in their host plants which is subsequently attenuated. We have recently obtained evidence that the MHB inoculation may lead to the attenuation of plant defense response in Norway spruce prior to fungal colonisation (N. Lehr et al., unpublished). The inoculation of spruce roots with *Streptomyces* sp. AcH 505 led to decreased peroxidase activities and gene expression levels in roots, markers for a defense response in spruce seedlings (Asiegbu et al. 1993; Fossdal et al. 2001). Simultaneously the colonization of roots by *Heterobasidion abietinum* 331 was promoted by AcH 505, although mycelial extension of this fungal strain was not affected by AcH 505. This suggests that AcH 505 promotes plant root colonization by fungi. Whether this indeed results from the production of unknown bacterial factors that suppress plant defense response remains to be investigated.

3.6 Fungus Specificity

Specificity in MHB-mycorrhizal fungus interactions was already indicated in early studies, which described bacterial species that promote and others that were either neutral or inhibitory to mycorrhiza formation (Garbaye and Bowen 1987, 1989). Frey-Klett et al. (2005) demonstrated that the Douglas fir–*L. bicolor* mycorrhizas and ectomycorrhizosphere selected *P. fluorescens* isolates that inhibited the mycelial growth of a larger range of phytopathogens in vitro than bulk soil isolates. In regard to ECM formation, they could demonstrate that there was a significantly higher proportion of ECM formation-inhibiting bacteria in the bulk soil zone in comparison with the symbiotic area. Fungus specificity has been addressed in several studies by Garbaye and co-workers. Garbaye and Duponnois (1992) showed that mycorrhiza formation of Douglas fir with *L. laccata* and some related *Laccaria* species was enhanced in the presence of MHB from *L. laccata* fruitbodies or Douglas fir–*L. bicolor* ECM whereas the establishment of the symbiosis with other fungi was inhibited. Two bacteria, *Pseudomonas fluorescens* BBc6 and *Pseudomonas* sp. SBc5, had a positive effect on *L. laccata* (from which they were isolated) and *L. bicolor,* and a negative effect on *Hebeloma cylindrosporum* and *Paxillus involutus*. Duponnois et al. (1993) reasoned that because of their selectivity,

MHBs might be an interesting, cheaper and safer alternative to soil fumigation. In a nursery experiment, the selectivity of two of these bacteria was confirmed; in a methyl bromide fumigated nursery soil, the MHB strains *Pseudomonas* sp. SBc5 and *P. fluorescens* BBc6 markedly improved the efficiency of the inoculation by *L. laccata* and closely-related species, but suppressed the mycorrhization of *P. menziensii* with *H. cylindrosporum* (Duponnois et al. 1993). Seven Western Australian bacterial isolates from *Laccaria fraterna* sporocarps or ectomycorrhizas, as well as *P. fluorescens* BBc6 and *Bacillus subtilis* MB3, were tested for their influence on ECM development of *Eucalyptus diversicolor* seedlings with three *Laccaria* spp. (Dunstan et al. 1998). Mycorrhiza formation by *L. fraterna* increased significantly with the indigenous isolates *Bacillus* sp. Elf28 and *Pseudomonas* sp. Elf29 and with the strains BBc6 and MB3. However, co-inoculation with the Australian *L. laccata* strain and the MHB isolate *P. fluorescens* BBc6 resulted in significantly inhibited ECM development. This was in stark contrast to the data from Duponnois and Garbaye (1991), who observed a significant promotion of ECM formation between a French *L. laccata* isolate and Douglas fir. Dunstan et al. (1998) suggested that this "fungal isolate specificity" reflects the genetic distance between the French and Australian *L. laccata* isolates.

Clear evidence for a fungus specificity factor came up recently, when the two antibiotics WS-5995 B and C were isolated from the culture supernatant of *Streptomyces* sp. AcH 505 (Riedlinger et al. 2006; Fig. 2b). The growth of *A. muscaria* was inhibited by micromolar concentrations of these substances, WS-5995 B being more effective than WS-5995 C. The production rate of the antibiotics by the bacterium ranged from 10 nM to 1 µM, and in co-culture with *A. muscaria*, down-regulation of WS-5995 B and C production to low nM levels was observed, due to the acidification of the culture medium (Riedlinger et al. 2006). The ECM fungus *Hebeloma cylindrosporum* which was suppressed in its growth in co-culture with *Streptomyces* AcH 505 was more sensitive to WS-5995 B than *A. muscaria*, promoted through this streptomycete. This indicates that the resistance towards WS-5995 B/C serves as a determining factor for fungus specificity by AcH 505 (Fig. 2a). Three genes in *A. muscaria* were observed to be upregulated after a treatment with WS-5995 B: a cell growth related aceto-acyl Coenzyme A synthetase (*AmAacs)*, a cell growth and cell stress related cyclophilin (*AmCyp40*), and a gamma-amino butyric acid/polyamine transporter (*Uga4)*. Riedlinger et al. (2006) speculated that *AmAacs* may be upregulated as a result of membrane damage, and *AmCyp40* and *Uga4* due to cell stress posed by the treatment with the antibiotic. The latter hypothesis was supported by increased gamma-amino butyric acid (GABA) levels in WS-5995 B treated hyphae, since GABA catabolism is involved in scavenging reactive oxygen species (Coleman et al. 2001). This suggests that WS-5995 B could cause oxidative stress and membrane damage in fungal hyphae, and that *A. muscaria* is either able to deal with these adverse effects on fungal physiology or to prevent the transfer of WS-5995 B into the fungal hyphae, to detoxify the substance, to export it or to transfer it into the vacuole. If any of these parameters were more pronounced in *A. muscaria* than in *H. cylindrosporum*, they could explain the fungus specificity of AcH 505.

Garbaye et al. (1992) demonstrated a mycorrhiza helper effect which was not specific to the host plant. Indeed, he showed that the helper effect, observed with the conifer Douglas fir, may be reproduced using deciduous tree species. When oak seedlings *(Quercus robur)* were inoculated with *L. laccata* and two helper pseudomonad isolates from Douglas fir–*L. laccata* mycorrhizas, both helper strains significantly increased the mycorrhiza formation in/on? the oak seedlings. The significant promotion of the formation of eucalyptus–*L. laccata* mycorrhizas by a MHB isolate from Douglas fir–*L. laccata* mycorrhizas (Dunstan et al. 1998) also supports this conclusion.

4 Potential for Use of Mycorrhiza Helper Bacteria in Agri- and Silviculture

Apart from the positive influence of MHB on mycorrhiza formation, stimulation of plant nutrition, growth, and suppression of phytopathogens by the helper bacteria have been observed. In the following, potential applications are discussed in the light of MHB research.

4.1 Plant Growth Promoting Helper Bacteria

Plant growth promoting bacteria (PGPB) exert their functions through mineral weathering (Calvaruso et al. 2006) mineralisation, plant hormone production and biological control (Barea et al. 2005). Several reports state that combined inoculation with PGPB and mycorrhizal fungi may yield synergistic positive effects on plant growth. The inoculation with *Azospirillum brasilense* and the AM fungi *Gigaspora margarita* or *Glomus fasciculatum* led to increased shoot and root biomass in pearl millet (*Pennisetum americanum*), due to improved phosphorus uptake (Rao et al. 1983). The MHB and PGPB *Pseudomonas fluorescens* 92 stimulates the growth of cucumber plants, and Gamalero et al. (2003) suggested that the reason for this was the strong rate of IAA production by the bacterium. Illustrating the complexity of the interactions within the mycorrhizosphere, Gamalero et al. (2004) showed that the use of two bacterial strains together with an AMF strongly improves tomato growth. The MHB strain *P. fluorescens* 92 was used together with the PGPB strain *P. fluorescens* P190r, and the combination of these two bacteria with *G. mossaeae* BEG12 led to strongly increased plant growth (Gamalero et al. 2004).

It is well known that mycorrhizosphere bacteria, including the MHB, may improve plant nutrition, and that P and N content of the soil affects the magnitude of plant responses to any microbial inoculation (Barea et al. 2005; Morgan et al. 2005). Barea and co-workers have found rhizobacteria that promote the establishment of AM and the solubilization of P from rock phosphate (Toro et al. 1997). The inoculation of onion with *Enterobacter* sp., *Bacillus subtilis* and AMF had a

positive influence on plant growth, P and N status. *B. subtilis* was especially effective. The inoculation of this bacterial isolate significantly increased AM symbiosis, shoot dry weight, shoot P content and shoot N content. These effects were at their greatest in dual inoculation experiments with the AM fungus *G. intraradices*, indicating an important role for the MHB effect in plant nutrition and growth. From a collection of *Rhizobium* strains native to the legume *Anthyllis cytisoides,* Requena et al. (1997) found *Rhizobium* strains that improve both AM establishment and the N status of *A. cytisoides*. The mycelium of the investigated AM fungi interacted differentially with the *Rhizobium* strains, whereas *G. intraradices* appeared to be more effective in P and N uptake with the *Rhizobium* strain NR 4, *G. coronatum* was more effective with the *Rhizobium* strain NR 9. Interestingly when these AM fungi were simultaneously co-inoculated with other *Rhizobium* and other rhizobacteria, the *Rhizobium* strain preferences of *G. intraradices* and *G. coronatum* were modulated. Therefore, these results indicate that a collection of native microbial isolates could be a good starting point for the selection of multifunctional microbe inocula for commercial purposes, but also underline the fact that it is difficult to predict the outcome of the interactions between plant beneficial microbes.

4.2 Phytoremediation or Increased Plant Survival in Polluted Soils

Microbial interactions to improve soil quality, remediation of polluted soils, have been addressed in several studies. In petroleum contaminated soils, Sarand et al. (1998) observed that the hyphal patches of the ECM fungus *Suillus bovinus* supported bacterial growth. These mycorrhizosphere bacteria were able to grow on media with toluate and xylene as sole sources, and to cleave catechol (Sarand et al. 1998). It should be tested if these bacteria, capable of mycorrhizo-degradation, could be mixed with MHB inocula to promote plant fitness in polluted soils. MHB increasing plant tolerance to Cd were characterized by Vivas et al. (2003a), and these were used as co-inoculants in remediation experiments. In a report on lead contaminated soils, Vivas et al. (2003d) showed that a *Brevibacillus* isolate was able to promote mycorrhization and nodulation, to decrease the amount of Pb absorbed by plants, and to improve shoot biomass, and N and P accumulation in *Trifolium pratense*. Due to the marked changes in root architecture, Vivas et al. (2003d) suggested that IAA production by the *Brevibacillus* isolate could be significant for the observed plant-beneficial capabilities.

4.3 Biocontrol and Controlled Mycorrhization

Root pathogens are a major concern in agriculture and forestry, and suppression of seedling death in nurseries is often not effective by conventional practices. Methods of biological control have, therefore, received increasing interest, including the use

of ECM and AM fungi against forest diseases (see reviews by Barea et al. 2005). The fungus specificity among the MHB indicates that the MHB could be used for a simultaneous promotion of certain symbiotic fungi and for the inhibition of plant pathogenic fungi. In vitro antagonism against phytopathogens has been frequently observed by MHB (Schelkle and Pererson 1996; Barea et al. 1998; Becker et al. 1999; Budi et al. 1999; Maier et al. 2004). With Norway spruce–*Heterobasidion annosum* pathosystem, we recently observed that *Streptomyces* sp. AcH 505 is antagonistic to against 11 of the 12 tested *Heterobasidion annosum* isolates. The antagonism led to a suppression of fungal colonization of Norway spruce roots and of agar covered wood disks. In contrast, mycelial growth rate of the 12th strain tested, *Heterobasidion abietinum* 331, was not affected by AcH 505, and the colonization of roots by this fungal strain was promoted by AcH 505. Bacterial inoculation led to decreased plant peroxidase activities and gene expression levels in roots. With these results it may be predicted that AcH 505 generally promotes plant root colonisation by fungi, restricted to fungal strains that are tolerant to antifungal metabolites produced by the bacterium (Fig. 2a). This indicates that fungus specificity and stimulated root receptivity by a MHB should be regarded as a potential risks in regard to biocontrol efficacy.

4.4 Persistence in the Soil and Dose-Dependent Effect on Mycorrhization

For the applications of MHB in nurseries and in the field it would be desirable that the bacteria would have a strong short-term influence on mycorrhization, but a minimal effect on native microbial populations. Frey-Klett et al. (1997) measured the development of MHB *P. fluorescens* BBc6 populations in nursery soil under greenhouse conditions. They showed a positive effect of bacterial inoculation on the Douglas fir- *L. bicolor* symbiosis in spite of the apparent survival of the bacterium of only 19 weeks in nursery soil. After a 4-year experiment in a forest plantation, no further effect on the MHB on mycorrhization was observable (Heinonsalo et al. 2004). Toro et al. (1997) inoculated phosphate solubilising rhizobacteria with AM fungi to onion seedlings and detected a similar drop in bacterial numbers as did Frey-Klett et al. (1997). After 60 days, the density of bacteria was dropped from 10^7 cfu g^{-1} of dry rhizosphere soil to 10^3 cfu g^{-1}. Still, highly significant positive effects on symbiosis development, seedling growth, N, and P contents were observed (Toro et al. 1997).

The effective dose of MHB that has to be used for increased mycorrhization varies between bacteria. Aspray et al. (2006) used *Pinus sylvestris–Lactarius rufus* symbiosis to test dose-effects with two MHB strains, *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67. Whereas EJP73 promoted mycorrhization at all doses tested, EJP67 only stimulated mycorrhiza formation at a narrow range of inoculum densities. Frey-Klett et al. (1999) showed that the mycorrhiza helper *P. fluorescens* BBc6 promotes mycorrhization only at low population doses (10 cfu/cm^3 soil), and

suggested that this helper strain could have some detrimental effects toward the plant or the fungus at higher population densities.

5 Perspectives

The complexity of the interactions within the mycorrhizosphere (Frey-Klett et al. 2005) can be exploited to the benefit of plants especially by using combinations of PGPR, MHB and mycorrhizal fungi in horticulture and tree nurseries (Gamalero et al. 2004). Recent data from polluted soils (Vivas et al. 2003d) indicates that MHB, possibly in combination with soil detoxifying bacteria, can also be used to improve plant fitness in toxic soils and for phytoremediation.

Novel and specific targeting and visualizing techniques for the microorganisms have to be developed in order to obtain a better understanding of pre-symbiotic fungal growth and root colonization processes in relation with the density of viable MHB. This is especially important since the speed of bacterial spread on the root surfaces as well as the morphology of the bacterial colony depend on the bacterial isolate (Poole et al. 2001; Aspray et al. 2006). Specific localization techniques have already been developed for certain bacteria (Artursson et al. 2005; Rincon et al. 2005; Aspray et al. 2006), and are under development for mycorrhizal fungi (Grimaldi et al. 2005; Müller et al. 2006).

A recent analysis of fungal gene expression during interaction with a MHB (Schrey et al. 2005; Deveau et al. 2007) will soon be followed by detailed microarray studies, as the sequencing of mycorrhizal fungal (*Laccaria bicolor*, *Tuber borchii*) and plant (*Lotus japonicus*, *Medicago truncatula, Oryza sativa, Populus trichocarpa*) genomes is finished. We may in the near future be able to identify gene clusters that are linked to the MHB effect and fungus specificity. The next step would then be to target the genome of selected MHB.

The development of molecular methods should, however, not distract from the continuing importance of physiological and biochemical studies. Physiological data will not only be essential for the interpretation of data from future microarray analyses, but also for addressing the functional diversity of MHB. Perhaps one of the most challenging fields ahead is the isolation of effector molecules from the MHB (Xie et al. 1995; Riedlinger et al. 2006).

References

Abdel-Fattah GM, Mohamedin AH (2000) Interactions between a vesicular-arbuscular mycorrhizal fungus (*Glomus intraradices*) and *Streptomyces coelicolor* and their effects on sorghum plants grown in soil amended with chitin of brawn scales. Biol Fertil Soils 32:401–409

Ali NA, Jackson RM (1989) Stimulation of germination of spores of some ectomycorrhizal fungi by other micro-organisms. Mycol Res 93:182–186

Ames BN (1989) Mycorrhiza development in onion in response to chitin-decomposing actinomycetes. New Phytol 112:423–427

Artursson V, Finlay RD, Jansson JK (2005) Combined bromodeoxyuridine immunocapture and terminal-restriction fragment length polymorphism analysis highlights differences in the active soil bacterial metagenome due to *Glomus mosseae* inoculation or plant species. Environ Microbiol 7:1952–1966

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8: 1–10

Asiegbu F, Daniel G, Johansson M (1993) Studies on the infection of Norway spruce roots by *Heterobasidion annosum*. Can J Bot 71:1552–1561

Aspray TJ, Frey-Klett P, Jones JE, Whipps JM, Garbaye J, Bending GD (2006a) Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. Mycorrhiza 10.1007/s00572-006-0068-3 (in press)

Aspray TJ, Eirian Jones E, Whipps JM, Bending GD (2006b) Importance of mycorrhization helper bacteria cell density and metabolite localization for the *Pinus sylvestris–Lactarius rufus* symbiosis. FEMS Microbiol Ecol 56:25–33

Azcon-Aguilar C, Diaz-Rodriguez RM, Barea JM (1986) Effect of soil microorganisms on spore germination of the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. Trans Br Mycol Soc 86:337–340

Barbieri E, Potenza L, Rossi I, Sisti D, Giomaro G, Rossetti S, Beimfohr C, Stocchi V (2000) Phylogenetic characterization and *in situ* detection of a *Cytophaga–Flexibacter–Bacteroides* phylogroup bacterium in *Tuber borchii* Vittad ectomycorrhizal mycelium. Appl Environ Microbiol 66:5035–5042

Barbieri E, Gioacchini AM, Zambonelli A, Bertini I, Stocchi V (2005) Determination of microbial volatile organic compounds from *Staphylococcus pasteuri* against *Tuber borchii* using solid phase microextraction and gas chromatography/ion trap mass spectrometry. Rapid Comm Mass Spectrom 15:3411–3415

Barea JM, Andrade G, Bianciotto V, Dowling D, Lohrke S, Bonfante P, O'Gara F, Azcon-Aguilar C (1998) Impact on arbuscular mycorrhiza formation of pseudomonas strains used as inoculants for biocontrol of soil-borne fungal plant pathogens. Applied Environ Microbiol 64:2304–2307

Barea JM, Pozo MJ, Azcon R, Azcon-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Becker DM, Bagley ST, Podila GK (1999) Effects of mycorrhiza-associated streptomycetes on growth of *Laccaria bicolor, Cenococcum geophilum*, and *Armillaria* species and on gene expression in *Laccaria bicolor*. Mycologia 91:33–40

Bedini, S, Bagnoli G, Sbrana C, Leporini C, Tola E, Dunne C, Filippi C, D'Andrea F, O'Gara F, Nuti MP (1999) Pseudomonads isolated from within fruit bodies of *Tuber borchii* are capable of producing biological control or phytostimulatory compounds in pure culture. Symbiosis 26:223–236

Bending GD, Poole EJ, Whipps JM, Read DJ (2002) Characterisation of bacteria from *Pinus sylvestris–Suillus luteus* mycorrhizas and their effects on root–fungus interactions and plant growth. FEMS Microbiol Ecol 39:219–227

Bertaux J, Schmid M, Prevost-Boure NC, Churin JL, Hartmann A, Garbaye J, Frey-Klett P (2003) *In situ* identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N. Appl Environ Microbiol 69:4243–4248

Bianciotto V, Bonfante P (2002) Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocelluar bacteria. Antonie Van Leeuwenhoek Int J Gen Mol Microbiol 81:365–371

Bianciotto V, Bandi C, Minerdi D, Sironi M, Tichy HV, Bonfante P (1996) An obligately endosymbiotic mycorrhizal fungus itself harbors obligately intracellular bacteria. Appl Environ Microbiol 62:3005–3010

Bowen GB (1993) The ecology of ectomycorrhiza formation and functioning. Plant Soil 159:61–67

Bowen GD, Theodorou C (1979) Interactions between bacteria and ectomycorrhizal fungi. Soil Biol Biochem 11:119–126

Brulé C, Frey-Klett P, Pierrat JC, Courrier S, Gérard F, Lemoine MC, Rousselet JL, Sommer J, Garbaye J (2001) Survival in the soil of the ectomycorrhizal fungus *Laccaria bicolor* and effect of a mycorrhiza helper *Pseudomonas fluorescens*. Soil Biol Biochem 33: 1683–1694

Budi SW, van Tuinen D, Martinotti MG, Gianinazzi S (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soil-borne fungal pathogens. Appl Environ Microbiol 65:5148–5150

Calvaruso C, Turpault MP, Frey-Klett P (2006) Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. Applied and Environmental Microbiology 72:1258–1266

Coleman ST, Fang TK, Rovinsky SA, Turano FJ, Moye-Rowley WS (2001) Expression of a glutamate decarboxylase homologue is required for normal oxidative stress tolerance in *Saccharomyces cerevisiae*. J Biol Chem 276:244–250

Danell E, Alström S, Ternström A (1993) *Pseudomonas fluorescens* in association with fruit bodies of the ectomycorrhizal mushroom *Cantharellus cibarius*. Mycol Res 97:1148–1152

De Oliveira VL, Garbaye J (1989) Les microorganismes auxiliaires de l'etablissement des symbioses ectomycorrhiziennes. Eur J For Pathol 19:54–64

Deveau A, Palin B, Delaruelle C, Peter M. Kohler A, Pierrat JC, Sarniguet A, Garbaye J, Martin F, Frey-Klett P (2007) The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N. New Phytologist 175:743–755

Dunstan WA, Malajczuk N, Dell B (1998) Effects of bacteria on mycorrhizal development and growth of container grown *Eucalyptus diversicolor* F. Muell. seedlings. Plant Soil 201:241–249

Duponnois R (2006) Bacteria helping mycorrhiza development. In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere. Springer, Heildelberg, pp 297–310

Duponnois R, Garbaye J (1990) Some mechanisms involved in growth stimulation of ectomycorrhizal fungi by bacteria. Can J Bot 68:2148–2152

Duponnois R, Garbaye J (1991) Mycorrhization helper bacteria associated with the Douglas fir *Laccaria laccata* symbiosis: effects in vitro and in glasshouse conditions. Ann Sci For 48:239–251

Duponnois R, Kisa M (2006) The possible role of trehalose in the mycorrhiza helper effect. Can J Bot 84:1005–1008

Duponnois R, Garbaye J, Bouchard D, Churin JL (1993) The fungus-specificity of mycorrhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomycorrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata*. Plant Soil 157:257–262

Duponnois R, Assikbetse K, Ramanankierana H, Kisa M, Thioulouse J, Lepage M (2006) Litter-forager termite mounds enhance the ectomycorrhizal symbiosis between *Acacia holosericea* A. Cunn. Ex G. Don and *Scleroderma dictyosporum* isolates. FEMS Microbiol Ecol 56:292–303

Fossdal CG, Sharma P, Lönneborg A (2001) Isolation of the first putative peroxidase cDNA from a conifer and the local and systemic accumulation of related proteins upon pathogen infection. Plant Mol Biol 47:423–435

Foster RC, Marks GC (1966) The fine structure of the mycorrhizas of *Pinus radiata*. Australian J Biol Sci 19:1027–1038

Founoune H, Duponnois R, Ba AM, Sall S, Branget I, Lorquin J, Neyra M, Chotte JL (2002) Mycorrhiza helper bacteria stimulated ectomycorrhizal symbiosis of *Acacia holosericea* with *Pisolithus alba*. New Phytol 153:81–89

Frey-Klett P, Garbaye J (2005) Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal-bacterial interactions. New Phytol 168:4–8

Frey-Klett P, Pierrat JC, Garbaye J (1997) Location and survival of mycorrhiza helper *Pseudomonas fluorescens* during establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir. Appl Environ Microbiol 63:139–144

Frey-Klett P, Churin J-L, Pierrat J-C, Garbaye J (1999) Dose effect in the dual inoculation of an ectomycorrhizal fungus and a mycorrhiza helper bacterium in two forest nurseries. Soil Biol Biochem 31:1555–1562

Frey-Klett P, Chavatte M, Clausse M-L, Courrier S, Le Roux C, Raaijmakers J, Martinotti MG, Pierrat J-C, Garbaye J (2005) Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytol 165:317–328

Frey-Klett P, Garbaye J, Tarkka M (2007) Tansley Review: The mycorrhiza helper bacteria revisited. New Phytologist 176:22–36

Fries N (1987) Ecological and evolutionary aspects of spore germination in the higher basidiomycetes. Trans Br Mycol Soc 88:1–7

Gamalero E, Fracchia L, Cavaletto M, Garbaye J, Frey-Klett P, Varese GC, Martinotti MG (2003) Characterization of functional traits of two fluorescent pseudomonads isolated from basidiomes of ectomycorrhizal fungi. Soil Biol Biochem 35:55–65

Gamalero E, Trotta A, Massa N, Copetta A, Martinotti MG, Berta G (2004) Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14:229–234

Garbaye J (1991) Biological interactions in the mycorhizospere. Experientia 47:370–375

Garbaye J (1994) Mycorrhiza helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Garbaye J, Bowen GD (1987) Effect of different microflora on the success of ectomycorrhizal inoculation of *Pinus radiata*. Can J For Res 17:941–943

Garbaye J, Bowen GD (1989) Stimulation of mycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. New Phytol 112:383–388

Garbaye J, Churin JL, Robin Duponnois R (1992) Effects of substrate sterilization, fungicide treatment, and mycorrhization helper bacteria on ectomycorrhizal formation of pedunculate oak (*Quercus robur*) inoculated with *Laccaria laccata* in two peat bare-root nurseries. Biol Fertil Soils 13:55–57

Garbaye J, Churin JL, Robin Duponnois R (1992) Effects of substrate sterilization, fungicide treatment, and mycorrhization helper bacteria on ectomycorrhizal formation of pedunculate oak (*Quercus robur*) inoculated with *Laccaria laccata* in two peat bare-root nurseries. Biol Fertil Soils 13:55–57

Garbaye J, Duponnois R (1992) Specificity and function of mycorrhization helper bacteria (MHB) associated with the *Pseudotsuga menziesii-Laccaria laccata* symbiosis. Symbiosis 14:335–344

Gazzanelli G, Malatesta M, Pianetti A, Baffone W, Stocchi V, Citterio B (1999) Bacteria associated to fruit bodies of the ectomycorrhizal fungi *Tuber borchii* Vittad. Symbiosis 26:211–222

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65: 5571–5575

Grimaldi B, de Raaf MA, Filetici P, Ottonello S, Ballario P (2005) Agrobacterium-mediated gene transfer and enhanced green fluorescent protein visualization in the mycorrhizal ascomycete *Tuber borchii*: a first step towards truffle genetics. Curr Genet 48:69–74

Gryndler M, Vosatka M (1996) The response of *Glomus fistulosum*-maize mycorrhiza to treatments with culture fractions from *Pseudomonas putida*. Mycorrhiza 6:207–211

Heinonsalo J, Frey-Klett P, Pierrat J-C, Churin J-L, Vairelles D, Garbaye J (2004) Fate, tree growth effect and potential impact on soil microbial communities of mycorrhizal and bacterial inoculation in a forest plantation. Soil Biol Biochem 36:211–216

Helber N, Requena N (2008) Expression of the fluorescence markers DsRed and GFP fused to a nuclear localization signal in the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 177:537–548

Hildebrandt U, Janetta K, Bothe H (2002) Towards growth of arbuscular mycorrhizal fungi independent of a plant host. Appl Environ Microbiol 68:1919–1924

Hildebrandt U, Ouziad F, Marner FJ, Bothe H (2006) The bacterium *Paenibacillus validus* stimulates growth of the arbuscular mycorrhizal fungus *Glomus intraradices* up to the formation of fertile spores. FEMS Microbiol Lett 254:258–267

Keller S, Schneider K, Sussmuth RD (2006) Structure elucidation of auxofuran, a metabolite involved in stimulating growth of fly agaric, produced by the mycorrhiza helper bacterium *Streptomyces* AcH 505. J Antibiot (Tokyo) 59:801–803

Krishna KR, Balakrishna AN, Bagyaraj DJ (1982) Interaction between a vesicular arbuscular mycorrhizal fungus and *Streptomyces cinnamomeus* and their effects on finger millet. New Phytol 93:401–405

Lagrange H, Jay-Allgmand C, Lapeyrie F (2001) Rutin, the phenylglycoside from eucalyptus root exudates, stimulates *Pisolithus* hyphal growh at picomolar concentrations. New Phytol 149:349–355

Lehr NA, Schrey SD, Bauer R, Hampp R, Tarkka MT (2007) Suppression of plant defense response by a mycorrhiza helper bacterium. New Phytol 174:892–903

Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora - the mycorrhizosphere effect. Phytopathology 78:366–371

Maier A (2003) Einfluss bakterieller Stoffwechselprodukte auf Wachstum und Proteom des Ektomykorrhizapilzes *Amanita muscaria*. PhD thesis, University of Tübingen, Tübingen, Germany

Maier A, Riedlinger J, Fiedler H-P, Hampp R (2004) Actinomycetales bacteria from a spruce stand: characterization and effects on growth of root symbiotic, and plant parasitic soil fungi in dual culture. Mycol Progress 3:129–136

Marschner P, Timonen S (2006) Bacterial community composition and activity in rhizospheres of roots colonised by arbuscular mycorrhizal fungi. In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere Springer, Heidelberg, pp 139–154

Mayo K, Davis R, Motta J (1986) Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431

Meyer JR, Linderman RG (1986) Response of subterranean clover to dual inoculation with vesicular-arbuscular fungi and a plant growth promoting bacterium. Soil Biol Biochem 18:185–190

Mogge B, Loferer C, Agerer R, Hutzler P, Hartmann A (2000) Bacterial community structure and colonization patterns of *Fagus sylvatica* L. ectomycorrhizospheres as determined by fluorescence *in situ* hybridization and confocal laser scanning microscopy. Mycorrhiza 9:271–278

Morgan JAW, Bending GD, White PJ (2005) Biological costs and benefits to plant–microbe interactions in the rhizosphere. J Exp Bot 56:1729–1739

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mugnier J, Mosse B (1987) Spore germination and viability of a vesicular-arbuscular mycorrhizal fungus, *Glomus mosseae*. Trans Br Mycol Soc 88:411–413

Muller T, Benjdia M, Avolio M, Voigt B, Menzel D, Pardo A, Frommer WB, Wipf D (2006) Functional expression of the green fluorescent protein in the ectomycorrhizal model fungus *Hebeloma cylindrosporum*. Mycorrhiza 16:437–442

Paula MA, Urquiaga S, Siqueira JO, Döbereiner J (1992) Synergistic effects of vesicular-arbuscular mycorrhizal fungi and diazotrophic bacteria on nutrition and growth of sweet potato (*Ipomoea batatas*). Biol Fertil Soils 14:61–66

Poole EJ, Bending GD, Whipps JM, Read DJ (2001) Bacteria associated with *Pinus sylvestris-Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*. New Phytol 151:743–751

Rao NSS, Tilak KVBR, Singh CS (1985) Synergistic effect of vesicular-arbuscular mycorrhizae and *Azospirillum brasiliense* on the growth of barley in pots. Soil Biol Biochem 1:121–129

Reddy MS, Satyanarayana T (2006) Interactions between ectomycorrhizal fungi and rhizospheric microbes In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere. Springer, Heidelberg, pp 245–264

Requena N, Jimenez I, Toro M, Barea JM (1997) Interactions between plant growth promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. New Phytol 136:667–677

Riedlinger J (2006) Die stofflichen Grundlagen der Modulation des Myzelwachstums von symbiotischen und pathogenen Pilzen durch Streptomyzeten. PhD thesis, University of Tübingen, Tübingen, Germany

Riedlinger J, Schrey SD, Tarkka MT, Hampp R, Kapur M, Fiedler HP (2006) Auxofuran, a novel substance stimulating growth of fly agaric, produced by the mycorrhiza helper bacterium Streptomyces AcH 505. Appl Environ Microbiol 72:3550–3557

Rincon A, Ruiz-Diez B, Garcia-Fraile S, Garcia JA, Fernandez-Pascual M, Pueyo JJ, de Felipe MR (2005) Colonisation of *Pinus halepensis* roots by *Pseudomonas fluorescens* and interaction with the ectomycorrhizal fungus *Suillus granulatus*. FEMS Microbiol Ecol 51:303–311

Sarand I, Timonen S, Nurmiaho-Lassila E-L, Koivula T, Haahtela K, Romantschuk M, Sen R (1998) Microbial biofilms and catabolic plasmid harbouring degradative fluorescent pseudomonads in Scots pine mycorrhizospheres developed on petroleum contaminated soil. FEMS Microbiol Ecol 27:112–115

Schelkle M, Peterson RL (1996) Suppression of common root pathogens by helper bacteria and ectomycorrhizal fungi *in vitro*. Mycorrhiza 6:481–485

Schrey SD, Schellhammer M, Ecke M, Hampp R, Tarkka MT (2005) Mycorrhiza helper bacterium *Streptomyces* AcH 505 induces differential gene expression in the ectomycorrhizal fungus *Amanita muscaria*. New Phytol 168:205–216

Schrey SD, Salo V, Raudaskoski M, Hampp R, Nehls U, Tarkka MT (2007) Interaction with mycorrhiza helper bacterium *Streptomyces* sp. AcH 505 modifies organisation of actin cytoskeleton in the ectomycorrhizal fungus *Amanita muscaria* (fly agaric). Curr Genet 52:77–85

Tarkka MT, Schrey S, Nehls U (2006) The alpha-tubulin gene *AmTuba1*: A marker for rapid mycelial growth in the ectomycorrhizal basidiomycete *Amanita muscaria*. Curr Genet 49:294–301

Toro M, Azcon R, Barea JM (1997) Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate- solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl Environ Microbiol 63:4408–4412

Torsvik, V, Salte, K, Sorheim, R, Goksoyr, J (1990) Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Appl Environ Microbiol 56:776–781

Vivas A, Azcón R, Biró B, Barea JM, Ruiz-Lozano JM (2003a) Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J Microbiol 49:577–588

Vivas A, Marulanda A, Gomez M, Barea JM, Azcon R (2003b) Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels. Soil Biol Biochem 35:987–996

Vivas A, Marulanda A, Ruiz-Lozano JM, Barea JM, Azcon R (2003c) Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza 13:249–256

Vivas A, Voros I, Biro B, Campos E, Barea JM, Azcon R (2003d) Symbiotic efficiency of autochthonous arbuscular mycorrhizal fungus (*G. mosseae*) and *Brevibacillus* sp. isolated from cadmium polluted soil under increasing cadmium levels. Environ Pollut 126:179–189

Vivas A, Barea JM, Azcon R (2005) *Brevibacillus brevis* isolated from cadmium- or zinc-contaminated soils improves in vitro spore germination and growth of *Glomus mosseae* under high Cd or Zn Concentrations. Microbial Ecol 49:416–424

Vosatka M, Gryndler M (1999) Treatment with culture fractions from *Pseudomonas putida* modifies the development of *Glomus fistulosum* mycorrhiza and the response of potato and maize plants to inoculation. Appl Soil Ecol 11:245–251

Wilkinson KG, Dixon KW, Sivasithamparam K (1989) Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation to germination of Australian orchids. New Phytol 112:429–435

Wilkinson KG, Dixon KW, Sivasithamparam K, Ghisalberti EL (1994) Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria. Plant Soil 159:291–295

Will ME, Sylvia DM (1990) Interaction of rhizosphere bacteria, fertilizer, and vesicular-arbuscular mycorrhizal fungi with sea oats. Appl Environ Microbiol 56:2073–2079

Xavier LJC, Germida JJ (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Soil Biol Biochem 35:471–478

Xie ZP, Staehelin C, Vierheilig H, Wiemken A, Jabbouri S, Broughton WJ, Vogeli-Lange R, Boller T (1995) Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiol 108:1519–1525

Genomic Organization and Mechanisms of Inheritance in Arbuscular Mycorrhizal Fungi: Contrasting the Evidence and Implications of Current Theories

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1 Introduction

Most plants, including the majority of crop species, associate with a specific group of soil fungi called arbuscular mycorrhizal (AM) fungi. These fungi facilitate the uptake of plant resources and are increasingly acknowledged as being critical to ecosystem function. This high level of ecological success is not expected for species that reproduce asexually, as they are expected to be vulnerable to accumulation of deleterious mutations (Muller 1932; Kondrashov 1988) and pathogenesis (Hamilton 1980; Lively 1987). Rather, AM fungi have flourished since the origin of plants (Pirozynski and Malloch 1975; Redecker et al. 2000), and appear to be the oldest asexual multicellular eukaryotes. While the ecological importance and evolutionary novelty of these fungi have become clear, the basic genetics of these fungi remain enigmatic. In fact, their genetic structure seems unusual in two ways. Firstly, individual cells always contain many nuclei, into the thousands or tens of thousands. And secondly, individual cells also contain very high levels of genetic variation, with as many as 13 variants at what would be expected to be single copy regions of the genome. It is likely that the presence and maintenance of this high level of standing variation is related to the long-term persistence and ecological success of these asexual species. However, basic issues such as the arrangement of this variation between, or within, nuclei remain controversial.

Two basic organizational structures have been advocated. Firstly, it is possible that all intra-cellular variation is present within individual nuclei and all of the nuclei within a cell are identical, i.e., homokaryotic (Pawlowska and Taylor 2004; Pawloska 2005). Alternatively, much of the genetic variation may be distributed between nuclei, with each cell containing multiple genomes, i.e., heterokaryotic (Bever and Morton 1999; Kuhn et al. 2001; Hijri and Sanders 2005). These two scenarios have very different implications for our understanding of inheritance of

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genetic variation and maintenance of this genetic variation in AM fungi. In this chapter, we explore the evidence for these two scenarios and their implications.

2 Mendelian versus Non-Mendelian Inheritance

The modern study of transmission genetics began with Mendel's investigation of the inheritance of variable characters in the domesticated pea (Mendel 1865). Mendel identified the central roles of segregation and sexual recombination in the transmission of particulate inheritance, and these discoveries have since been codified into one of the unifying principles of biology (Suzuki et al. 1986). The simple segregation and recombination patterns predicted by this mode of inheritance are well described by simple statistical distributions that then formed the foundation of the fields of population and statistical genetics. With these tools, we are able to project evolutionary dynamics into the future, and make inference into evolutionary events of the past (Weir 1996; Lynch and Walsh 1998).

Mendelian transmission, however, does not apply to all genetic systems. Mendel's rules have been found to describe transmission of nuclear genes during sexual reproduction of eukaryotes. Eukaryotes, however, also have important non-nuclear genetic systems in the form of genes in mitochondria and chloroplasts. The transmission of these genes does not follow the Mendelian paradigm and they are instead generally inherited solely from the maternal parent.

Non-Mendelian genetic systems are common in fungi, in particular. Many fungi have a prolonged and dominant haploid stage. These nuclei, however, can exist alone in a single cell (monokaryon) or multiple nuclei can co-habit a single cell (di- or multikaryon). These multinucleate stages may eventually proceed to sexual fusion (karyogamy) followed quickly by meiosis. However, for some fungi, the multinucleate stages are long-lived and stable. These multinucleate stages represent interesting challenges as genetic systems. In Basidiomycetes and Ascomycetes, dikaryotic cells are formed by fusion of haploid hyphae during mating prior to meiosis (Davis 1966). Often, the hyphae are from similar "compatibility groups", but the rules for hyphal fusion can be complex. These multinucleate hyphae can be heterokaryotic (i.e., including genetically diverse nuclei). The heterokaryons can fuse with additional monokaryons or other heterokaryons and, conversely, a heterokaryon hypha can lose nuclei or bud-off monokaryons. In addition, there can be occasional non-meiotic recombination of genetic material between cohabitating nuclei within heterokaryons (i.e., parasexuality, Leslie 1993; Alexopoulos et al. 1996). Such unusual genetic processes defy easy statistical description of their transmission properties.

Genetic systems of fungi not only present challenges in understanding inheritance, but simultaneously present novel problems regarding the translation of genotype to phenotype. For example, how does the genetic material in different nuclei of a heterokaryon interact to produce a phenotype? There is much evidence of genetic complementation of mutations between nuclei cohabitating a heterokaryon. For example, two mutants of *Neurospora crassa*, one lacking the ability to synthesize

p-maniobenzoic acid and the other nicotinic acid, are able to grow as a heterokaryon on media that would otherwise inhibit either monokaryons (Ingold and Hudson 1993). This provides evidence that the phenotype can reflect the collective genotype of the multinucleate hyphae.

3 AM Fungal Cells Harbor Many Nuclei and Many Genetic Variants

The genomic structure of AM fungi is unusual in at least two respects. First, AM fungi are multinucleate at all stages of their life history. Individual cells may contain as many as a few hundreds to tens of thousands of nuclei depending on the fungal species and the method of analysis employed (Cooke et al. 1987; Becard and Pfeffer 1993; Hosny et al. 1998). As the AM fungal hyphae lack regular septa and the fungi do not appear to go through a uni-nucleate or sexual stage, the vegetative structures can be thought of as free-flowing populations of nuclei.

The second unusual aspect of their genetics is that individual cells can have very large amounts of genetic variation, with repetitive regions such as ribosomal RNA genes (rDNA) having several genetically different copies derived from single spores (Sanders et al. 1995; Lloyd-MacGilp et al. 1996; Hijri et al. 1999; Clapp et al. 1999; Pringle et al. 2000; Pawlowska and Taylor 2004). While a component of this considerable variation has been found to be due to non-mycorrhizal fungi that cohabit with, and contaminate, AM fungi (Hijri et al. 2002), these contaminants do not negate the high diversity of rDNA of AM fungal origin (Pringle et al. 2003). Moreover, a similar level of variation within spores has been observed within single copy regions of the genome (Kuhn et al. 2001; Pawlowska and Taylor 2004), with, for example, 13 different variants of putatively single copy gene, DNA polymerase 1 (PLS1), being found within individual spores of *Glomus etunicatum*. The processes maintaining the high intracellular genetic diversity may be critical to our understanding of the long-term persistence of these asexual lineages.

4 Alternative Hypotheses on Genomic Organization: The Evidence

The distribution of allelic diversity within the cells, as well as the genetic processes maintaining the variation, has been a matter of considerable dispute. The genetic diversity within single cells could be distributed within or among nuclei. At one extreme, all of the variation could be held within any one nucleus and all nuclei within a cell may then be identical (i.e., they are *homokaryotic*). Alternatively, nuclei within a cell could be genetically different (i.e., they are *heterokaryotic*), in which case there could be substantial variation between nuclei.

Over the last ten years, three types of evidence have arisen which inform this question: (1) attempts to directly score nuclei within hyphae, (2) tests of segregation of variants, and (3) tests of recombination between loci.

4.1 Evidence from Direct Scoring of Nuclei within AMF Cells

Hijri and colleagues (1999) attempted to score individual nuclei by serial dilution of an extract of nuclei derived from a single spore of *Scutellospora castanea*. A solution of nuclei was diluted until an estimated 43% of the total samples contained nuclei and 66% of these samples were expected to be harboring a single nucleus. Hijri and colleagues then amplified and scored the size of a region of rDNA that had been found to be polymorphic within a single spore. Individual amplification was found to contain different sized fragments, which is consistent with expectations from the original spore being heterokaryotic. With this design, there is a risk that DNA had broke free from nuclei before the dilution. However, this would have changed the expectations for the proportion of samples that contain nuclei, and the proportion observed was consistent with these expectations. Moreover, the control amplifications from multinuclear extractions consistently showed strong polymorphisms with the rDNA indicating that the results were not PCR artifacts.

Trouvelot and colleagues (1999) were the first to attempt to visualize different nuclei types within cells of AM fungi by developing markers of variation in rDNA and labeling the 28S rDNA markers using fluorescent tags. Spores were squashed, nuclei fixed to slides and the DNA labeled with fluorescent markers (via fluorescent DNA-DNA in situ hybridization (FISH)). Using this technique on spores of *S. castanea, Glomus intraradices, G. mosseae,* and *Gigaspora rosea*, they observed what appeared to be nuclei that harbored genetically different rDNA markers, indicating heterokaryotic nuclei within spores. This interpretation, however, is weakened by the fact that the annealing rate of the label was not controlled and low rates of labeling could generate the appearance of different nuclei types from nuclei that are in fact homokaryotic. Kuhn and coworkers (2001) again used the FISH approach to score DNA of spores of *S. castanea* labeling with two variants of the rDNA internal transcribed spacer sequences (ITS). They again found visual evidence consistent with genetically distinct nuclei being present within individual spores, as some spores were labeled by one of the two tags and other nuclei by both. The authors then developed a statistical argument in favor of heterokaryosis in which the frequency of observation of differently labeled nuclei exceeded that expected by observed frequency of labeling even if one allowed for substantial interference. Some authors, however, suggest caution in interpreting these results (Pawlowska 2005) because of the potential problems in interference of probes access to target DNA in FISH experiments can lead to discrepancies in signal detection (Yilmaz and Noguera 2004). However, the controls used for efficient probe penetration would appear to negate these suggestions, barring strong non-linearities (Kuhn et al. 2001).

Pawlowska and Taylor (2004) reported attempts to amplify rDNA from nuclei that they had dissected from a single spore of *G. etunicatum* and *G. intraradices*. They found that each of the dissected nuclei contain the same set of three ITS rDNA variants and they identified that this was consistent with a homokaryotic arrangement of molecular variants. However, Bever and Wang (2005) noted that the presence of the three types within a nucleus is not a definitive test for homokaryosis as the nuclei could still vary in the numbers for each of the three ITS types as well as in other regions of the genome. Pawlowska and Taylor (2005) suggested that changes in copy number of rDNA are not relevant, citing evidence that copy number can be dynamic within a cell cycle. While there can be rapid physiological accumulation of extra chromosomal elements of circular rRNA repeats (as cited by Pawlowska and Taylor) and these changes would not be relevant to considerations of inheritance, there is also much evidence of rapid evolution in the number of rRNA repeats within chromosomes of fungi (Zolan 1995), and this variation can be ecologically important.

4.2 Evidence from Patterns of Segregation

The homokaryotic versus heterokaryotic arrangements of genetic variation yield two different expectations for the segregation of this variation across generations. In the case of homokaryotic arrangement, all offspring are expected to have identical genetic composition to their own and to their parental spore. However, in the case of heterokaryotic arrangement of genetic variation, genetically different nuclei are expected to segregate out into different frequencies, leading to divergence of offspring hyphae. Over time, this process is expected to lead to the loss of variation from offspring spores (Bever and Morton 1999; Kuhn et al. 2001; Pawlowska and Taylor 2004; Hijri and Sander 2005).

Bever and Morton (1999) presented the first evidence for segregation of different nuclei types through observations of inheritance of spore shapes of *S. pellucida*. They found that replicate single spore isolates from a single population had different spore shapes and that the average spore shapes were highly heritable. Moreover, the cultures also differed in the variance, skewness and kurtosis of the distributions of spore shape (Fig. 1). These observations are difficult to explain under the homokaryotic hypothesis, as it would require positing that the nuclei in different spores differed in many ways, including maternal effects on the distribution of offspring spore shape. However, the results are very consistent with expectations from the heterokaryotic hypothesis. In the course of making this argument, Bever and Morton (1999) introduced a model of inheritance which we will review separately below. Pawlowska (2005) questioned this interpretation, suggesting that the different isolates may be different species. However, the morphological species concept within *Scutellospora* has been supported by both morphological and molecular evidence (Franke and Morton 1994; Morton 1995; Bentivenga and Morton 1996), and our analysis of rDNA sequences indicates that these isolates are indeed the same species (H. Kang, personal observation).

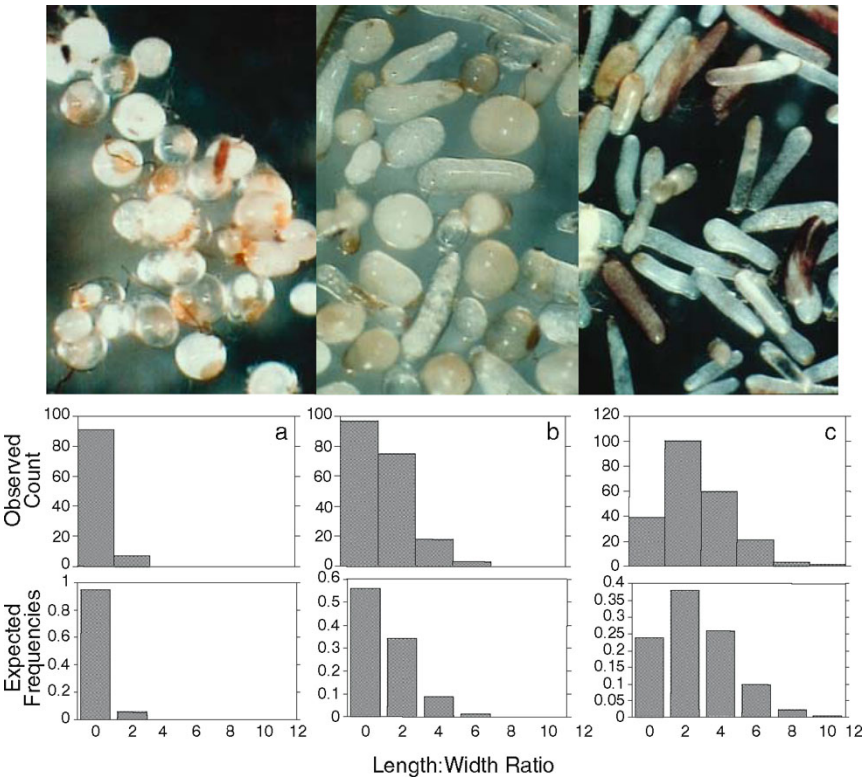

Fig. 1 Spores and spore shape distributions from three of the single spore isolates from Bever and Morton (1999). The *top histograms* represent the observed distribution of spore shape as the ratio of length over width. The *lower histograms* represent the expected distributions from the parameters fit by the model of inheritance described by Bever and Morton (1999)

Pawlowska and Taylor (2004) found that high levels of intra-cellular molecular variation within isolates of *G. etunicatum* were not lost due to segregation. They observed that each of 20 single progeny spores had all 13 variants of PLS1, a putatively single copy gene. They argued that the lack of loss of these variants was inconsistent with heterokaryotic organization of the genome, with their statistical confidence in this conclusion coming from simulations of the segregation process that assumed haploidy, no hyphal fusion and no selection. Instead, they proposed that all 13 variants of the PLS1 gene were present within each nucleus (and all nuclei in the hyphae were identical), with the persistence of the large number of variants within individual spores resulting from very high ploidy in these fungi (i.e., at least 13 ploids) and the suspension of gene conversion. This explanation came into conflict with the subsequent observation that *G. etunicatum* is actually haploid (Hijri and Sanders 2005).

Bever and Wang (2005) presented a resolution to this apparent conflict by using a simulation similar to that of Pawlowska and Taylor (2004) to demonstrate that modest

levels of hyphal fusion would allow remixing of the nuclei and reduce the effective rate of segregation to a level consistent with the Pawlowska and Taylor's laboratory observations. With sufficient rates of hyphal fusion, high levels of variation can be maintained within spores over long periods of time, which is consistent with Pawlowska and Taylor's field observations. Pawlowska and Taylor (2005) argued that there is no evidence of the level of hyphal fusion assumed in Bever and Wang's simulation, citing evidence of barriers to hyphal fusion between geographically isolated populations of *G. mosseae* (Giovannetti et al. 2003). However, this same body of work shows very high rates of hyphal fusion within isolates of many species of *Glomus* (Giovannetti et al. 1999, 2001, 2004). The simulation of Bever and Wang simply assumed that offspring from a single spore could fuse, which is exactly what had been demonstrated by the work of Giovannetti and others. Moreover, several studies of Giovannetti's group are of isolates derived from INVAM, which were derived from multiple spores (Morton et al. 1993), and other, similarly derived, INVAM cultures have been shown to be genetically heterogeneous populations (Bentivenga et al. 1997).

4.3 Evidence of Recombination from Field Samples

Tests of recombination can inform our understanding of mechanisms of inheritance in AM fungi by providing evidence for the frequency of genetic exchange. Genetic recombination could result from an unseen sexual stage (consistent with homokaryotic arrangement within hyphae), but it is more likely that such genetic exchange would result from nuclear exchange following hyphal fusion of genetically different hyphae (as is possible in the heterokaryotic model).

There have been several tests of recombination from multilocus datasets generated from field collected spores. Of these, one study found evidence of genetic exchange in two of the six unmanaged populations tested (Vanderkoornhuyse et al. 2001). This study, however, used loci identified by DNA fingerprinting and therefore the AM fungal origin of the different bands cannot be confirmed, giving rise to the possibility that the recombining bands represented patterns of infection of a contaminant micro-organism (Stuckenbrock and Rosendahl 2005; Pawlowska 2005).

A study by Stukenbrock and Rosendahl (2005) used three codominant genetic markers of confirmed AMF origin to estimate the genetic structure of two AM fungal populations from agricultural fields, and they did not find evidence of significant recombination. The clonal structure of these populations is not surprising given that the populations are continually disturbed due to cropping practice, and are likely to have been recolonized from neighboring populations, and genetically distinct initial founders of the population will generate high indices of association. Left undisturbed and assuming selective neutrality, hyphal fusion and nuclear mixing would be expected to reduce the indices of association overtime. Such decay association indices overtime would provide a much stronger test for remixing and should be the focus of future work in this area.

5 Alternative Hypotheses on Genomic Organization: The Implications

Individual scientists differ in their interpretations of the weight of available evidence on the genomic organization of AM fungi. Pawlowska (2005) is unconvinced by the work suggesting AM fungi are heterokaryotic and argues that the current work favors the homokaryotic hypothesis. While reviewing the same studies, we feel there is good evidence that AM fungi are heterokaryotic. However, at some level, the "homokaryotic" versus "heterokaryotic" dispute on the nature of AM fungal genomic structure could be regarded as simplistic. Given that AM fungi do not go through a life history stage involving a single nucleus per cell, then individual mutations will create differences between nuclei, thereby forcing heterokaryosis. The real issue then is not whether AM fungi are heterokaryotic, but to what extent are they heterokaryotic. This "degree of heterokaryosis" issue has important implications for our understanding of the evolutionary genetics of AM fungi.

5.1 Implications of Homokaryotic Genomic Organization

If AM fungi have large amounts of genetic variation contained within each nucleus, then this suggests that concerted evolution is unusually weak in these species (Pawlowska and Taylor 2004). Concerted evolution is a homogenizing force within multicopy gene families and results from infrequent unequal crossing over events occurring during mitosis and/or meiosis, which are followed by gene conversion. Over time, these processes can reduce variation within multicopy gene families, accounting for the low variation within rDNA gene families in other organisms (Hamby and Zimmer 1992; Avise 2004). It would be surprising if gene conversion were slowed in AM fungi, and would beg an explanation.

Furthermore, explanation for the long-term persistence of the asexual AM fungi in the face of mutation pressure is a challenge under the homokaryotic hypothesis. However, it is possible that the high redundancy due to polyploidy, combined with high hyphal population sizes, would slow the deleterious effects of mutation accumulation (Muller 1932) and thereby contribute to the longevity of these asexual lineages (Pawlowska and Taylor 2004).

5.2 Implications of Heterokaryotic Genomic Organization

If substantial amounts of variation are maintained between nuclei, we would need to identify what processes could contribute to the coexistence of multiple genomes, as nuclear segregation during hyphal growth and division would be expected to reduce nuclear diversity. There are several possibilities, including balancing selection and

constrained coordinated movement of nuclei. But perhaps the most straightforward mechanism is the possibility of occasional fusion of genetically differentiated hyphae.

Hyphal fusion has been observed in many different species of AM fungi. While hyphal fusion is inhibited in geographically divergent isolates, hyphal fusion rates can be very high for fungal isolates from the same proximity (Giovannatti et al. 1999, 2001; de la Providencia et al. 2005). Using a stochastic simulation of nuclear segregation, Bever and Wang (2005) demonstrated that modest rates of hyphal fusion can maintain high levels of nuclear variation within spores at equilibrium. In Fig. 2, we further demonstrate that, while very low levels of hyphal fusion (2%) are

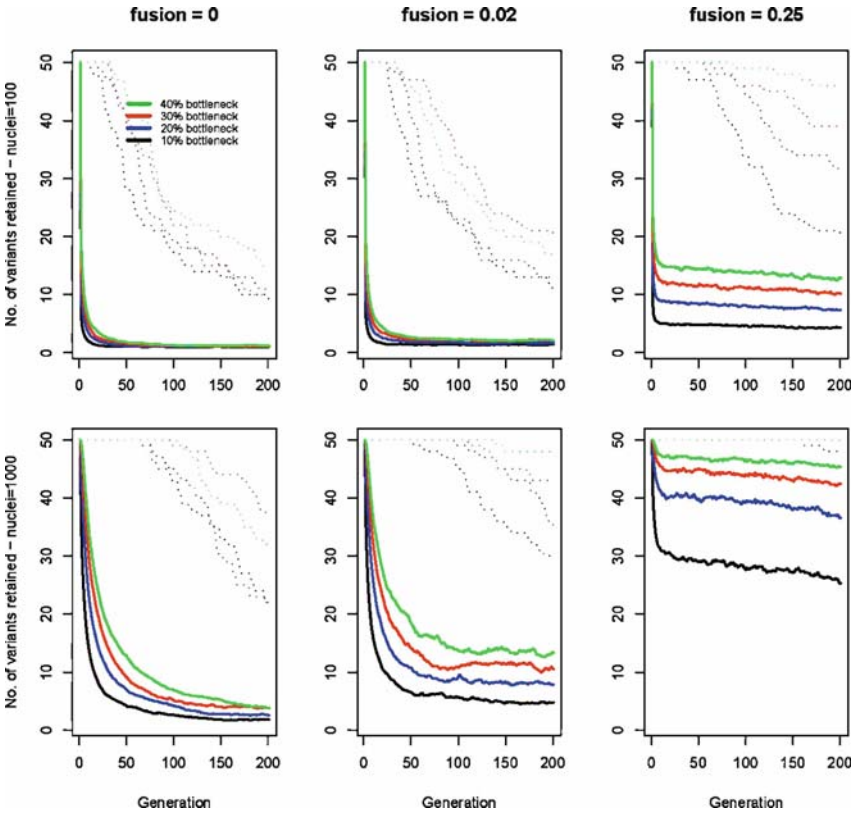

Fig. 2 We explored the implications of heterokaryotic genomic organization for the rate of decay of genetic diversity by simulating the progeny of a single spore that begins with 50 distinct nuclei types. Each generation, the nuclei are chosen from the parental population by chance at a sampling rate determined by the bottleneck rate (40, 30, 20 and 10%) with various combinations of the number of nuclei within the spores, the size of total spore population and the rate of hyphal fusion. The rate of decay in 200 generations is shown for population size of 1,000, numbers of nuclei within the spores of 100 and 1,000, and fusion rates of 0%, 2% and 25%. We monitored the decay of nuclear diversity within spores (*solid curves*) as well as the total nuclear diversity across the entire population (*dotted curves*). The results show that a little fusion goes a long way in maintaining the variants in fungi that have high numbers of nuclei

not sufficient to maintain genetic variation in hyphae that contain a hundred nuclei, these very low rates of hyphal fusion can have large effects in hyphae that contain a thousand nuclei. This result is significant as it matches with patterns of hyphal fusion within AM fungi. While smaller spored species within *Glomus* may have relatively small numbers of nuclei, which makes them very vulnerable to drift, *Glomus* generally have high rates of hyphal fusion which will reduce the rate of genetic drift (de la Providencia et al. 2005; Voets et al. 2006). In contrast, *Scutellospora* and *Gigaspora* generally appear to have lower rates of hyphal fusion (de la Providencia et al. 2005; Voets et al. 2006), but these fungi consistently have

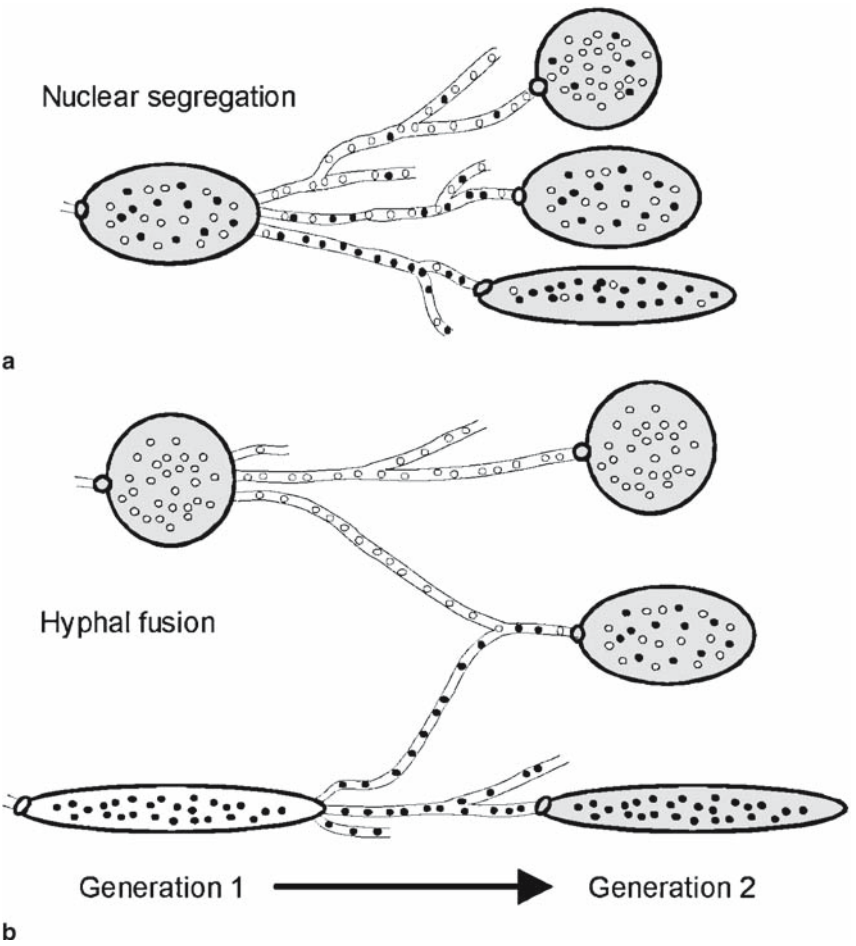

Fig. 3 Under heterokaryotic nuclear organization, the processes of nuclear segregation and hyphal fusion, (**a**) and (**b**), respectively, could segregate and remix variation in an analogous manner as meiosis and sexual gametic fusion. Spore shape is assumed to be a function of the proportion of filled nuclei (following Bever and Morton 1999)

larger numbers of nuclei with estimates ranging from a thousand to tens of thousands (Hosny et al. 1998). As our results show, with these large number of nuclei, the low rates of hyphal fusion can go a long way toward reducing the rate of drift (Fig. 2). These results suggest that hyphal fusion rates are sufficient to offset the force of drift in AM fungi, potentially providing an explanation for persistence of high levels of variation in AM fungal nuclei.

In this heterokaryotic scenario, alternative genetic processes could mimic the benefits of sexual recombination (Bever and Morton 1999). Variation that exists between nuclei would segregate as hyphae grow and divide (Fig. 3a), a process analogous to assortment during meiosis. Fusion of genetically different hyphae could remix and recombine variation (Fig. 3b) in an analogous manner as fusion of gametes in sexual organisms. Assuming that the phenotype is a function of the nuclear composition of the hyphae, this process could mimic the creative process of sexual reproduction by bringing together novel genetic variants into the same functional organism. Because the linkage groups are very large (i.e., contents of a nucleus), this process alone would not prevent the accumulation of deleterious mutations (Muller 1932). However, these processes combined with irregular parasexual recombination may minimize the accumulation of deleterious mutations.

6 A Model of Inheritance under Heterokaryosis

In the course of making an argument for the consistency of empirical observations of inheritance of spore shape in *S. pellucida* (Fig. 1), Bever and Morton (1999) proposed a simple model of inheritance under heterokaryosis. We highlight this model here as it provides insight into elements of inheritance when heterokaryotic nuclei are the source of phenotypic variation.

The segregation of heterokaryotic nuclei may be expected to generate an approximately Binomial or Multinomial distribution in offspring spores. If we imagine that two nuclei types control spore shape, one type coding for round spores and a second coding for oblong spores, the initial frequency of the oblong nuclei type would be identified as (p) in the parental spore. The rate of segregation of these nuclear types into offspring spores would be characterized by the effective number of these parental nuclei (n_e). In this model of inheritance, the key genetic parameter is n_e, as this value circumscribes the level of heterogeneity in the offspring distribution. The value of n_e will be a function of the rates of hyphal division (greater rates of division, lower n_e), rates of nuclear replication (higher rates of nuclear replication corresponds to higher average population sizes and higher n_e), and rates of hyphal fusion (higher rates of hyphal fusion will remix the nuclei, thereby raising n_e). These processes will determine how many parental nuclei will be represented in a given length of active fungal hyphae as the spore is produced.

A second set of assumptions is needed to describe the translation of hyphal genotype to phenotype. Work with ascomycetes show that genes from different nuclei can contribute to the phenotype of a hyphae (Ingold and Hudson 1993). We

assumed that there was a linear relationship between the nuclear content of the developing spore and the final shape of that spore (Bever and Morton 1999). With this model, we were able to estimate the genetic parameters, with which we could predict the expected offspring distributions. In Fig. 1, we illustrate both the observed distribution of spore shapes and the distribution of spore shapes predicted by a fit of the Binomial distribution to the data. The fit distributions are very close to the observed distributions and this close matching of the predicted and observed distributions provides support for the genetic model. From this, we suggest that the parental spores differed in their initial proportion of nuclei, with the round spore having a high abundance of nuclei coding for round shape and the oblong spores having a high abundance of nuclei coding for oblong shape. As the fungus grew (Fig. 3a), the nuclei multiplied and segregated into separate hyphae. This segregation resulted in lower variability in offspring spore shape in the lineage that was initially round (and very low p) and higher variability in the shape of offspring spores in the lineage with higher initial proportion of nuclei coding for oblong spores. In this way, the heritable differences in the mean, variance, skewness and kurtosis of spore shape may be the result of a single genetic mechanism: the segregation of heterokaryotic nuclei.

In this scenario, the segregation of nuclei alone would result in the fixation of nuclei types within spores. In fact, given our estimate of $n_e = 7$, we would expect to see noticeable fixation of lineages within our cultures. Instead, the distributions of spore shapes have been consistent over multiple generations, providing indirect evidence of an important role for hyphal fusion within these isolates.

Clearly, the utility of this model of inheritance needs to be tested for other traits and in other AM fungi. Should this model be supported, it may provide a means for gaining inference into patterns of evolution in a similar manner as Mendelian inheritance has allowed for many plants and animals (Weir 1996; Lynch and Walsh 1998).

7 Conclusions

Individual cells of AM fungi have high amounts of genetic variability and large numbers of nuclei. The arrangement of that genetic variation within or among these nuclei has been in dispute. While AM fungal hyphae are certainly heterokaryotic at some level due to the inevitability of mutation, the extent of inter-nuclear variation maintained within hyphae is difficult to assay. In reviewing the available evidence, we feel that there is strong support for substantial amounts of their genetic variation being maintained between nuclei. We then explored the theoretical implications of this arrangement, demonstrating that observed levels of hyphal fusion are sufficient to maintain observed levels of intra cellular variation in a heterokaryotic arrangement. We also present a simple model of inheritance with genetically divergent hyphae.

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References

Alexopoulos CJ, Mims CW, Blackwell M (1996) Introductory mycology, 4th edn. Wiley, New York

Avise J (2004) Molecular markers, natural history, and evolution, 2nd edn. Sinauer, Sunderland, Mass.

Becard G, Pfeffer PE (1993) Status of nuclear division in arbuscular mycorrhizal fungi during in-vitro development. Protoplasma 174:62–68

Bentivenga SP, Morton JB (1996) Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proc Nat Acad Sci USA 93:5659–5662

Bentivenga SP, Bever JD, Morton JB (1997) Genetic variation of morphological characters within a single isolate of the endomycorrhizal fungus *Glomus clarum* (Glomaceae). Am J Bot 84:1211–1216

Bever JD, Morton J (1999) Heritable variation and mechanisms of inheritance of spore shape within a population of *Scutellospora pellucida*, an arbuscular mycorrhizal fungus. Am J Bot 86:1209–1216

Bever JD, Wang M (2005) Arbuscular mycorrhizal fungi-hyphal fusion and multigenomic structure. Nature 433, E3–E4

Clapp JP, Fitter AH, Young JPW (1999) Ribosomal small subunit sequence variation within spores of an arbuscular mycorrhizal fungus, Scutellospora sp. Mol Ecol 8:915–921

Cooke JC, Gemma JN, Koske RE (1987) Observations of nuclei in vesicular-arbuscular mycorrhizal fungi. Mycologia 79:331–333

Davis RH (1966) Heterokaryosis, In: Ainsworth GS, Sussman AS (eds) The fungi: an advanced treatise. Academic, New York, pp 567–588

de la Providencia IE, de Souza FA, Fernandez F, Séjalon-Delmas N, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenetic groups. New Phytol 165:261–271

Franke M, Morton J (1994) Ontogenic comparisons of arbuscular mycorrhizal fungi *Scutellospora heterogama* and *Scutellospora pellucida* – revision of taxonomic character concepts, species descriptions, and phylogenetic hypotheses. Can J Bot 72:122–134

Giovannetti M, Azzolini D, Citernesi AS (1999) Anastomosis formation and nuclear and protoplasmic exchange in arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:5571–5575

Giovannetti M, Fortuna P, Citernesi AS, Morini S, Nuti MP (2001) The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol 151:717–724

Giovannetti M, Sbrana C, Strani P, Agnolucci M, Rinaudo V, Avio L (2003) Genetic diversity of isolates of Glomus mosseae from different geographic areas detected by vegetative compatibility testing and biochemical and molecular analysis. Appl Environ Microbiol 69:616–624

Giovannetti M, Sbrana C, Avio L, Strani P (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. New Phytol 164:175–181

Hamby RK, Zimmer EA (1992) Ribosomal RNA as a phylogenetic tool in plant systematics, In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York, pp 50–91

Hamilton WD (1980) Sex versus non-sex versus parasite. Oikos 35:282–290

Hijri M, Sanders IR (2005) Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. Nature 433:160–163

Hijri M, Hosny M, van Tuinen D, Dulieu H (1999) Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fungal Genet Biol 26:141–151

Hijri M, Redecker D, Petetot JAMC, Voigt K, Wostemeyer J, Sanders IR (2002) Identification and isolation of two ascomycete fungi from spores of the arbuscular mycorrhizal fungus *Scutellospora castanea*. Appl Environ Microbiol 68:4567–4573

Hosny M, Gianinazzi-Pearson V, Dulieu H (1998) Nuclear DNA content of 11 fungal species in Glomales. Genome 41:422–428

Ingold CT, Hudson HJ (1993) The biology of fungi, 6th edn. Chapman and Hall, London
Kondrashov AS (1988) Deleterious mutations and the evolution of sexual reproduction. Nature 336:435–440
Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414:745–748
Leslie JF (1993) Fungal vegetative compatibility. Annu Rev Phytopathol 31:127–150
Lively CM (1987) Evidence from a New Zealand snail for the maintenance of sex by parasitism. Nature 328:519–521
Lloyd-Macgilp SA, Chambers SM, Dodd JC, Fitter AH, Walker C, Young JPW (1996) Diversity of the ribosomal internal transcribed spacers within and among isolates of *Glomus mosseae* and related mycorrhizal fungi. New Phytol 133:103–111
Lynch M, Walsh JB (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland, Mass.
Mendel G (1865) Versuche über pflanzen-hybriden. Trans Verh Naturforsch Ver Brünn 4:3–270
Morton JB (1995) taxonomic and phylogenetic divergence among 5 *Scutellospora* species based on comparative developmental sequences. Mycologia 87:127–137
Morton JB, Bentivenga SP, Wheeler WW (1993) Germ plasm in the international collection of arbuscular and vesicular-arbuscular mycorrhizal fungi (INVAM) and procedures for culture development, documentation and storage. Mycotaxon 48:491–528
Muller HJ (1932) Some genetic aspectd of sex. Am Nat 66:118–138
Pawlowska TE (2005) Genetic processes in arbuscular mycorrhizal fungi. FEMS Microbiol Lett 251:185–192
Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737
Pawlowska TE, Taylor JW (2005) Arbuscular mycorrhizal fungi-hyphal fusion and multigenomic structure. Reply. Nature 433:E4–E5
Pirozynski KA, Malloch DW (1975) Origin of land plants-matter of mycotropism. Biosystems 6:153–164
Pringle A, Moncalvo JM, Vilgalys R (2000) High levels of variation in ribosomal DNA sequences within and among spores of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycologia 92:259–268
Pringle A, Moncalvo JM, Vilgalys R (2003) Revisiting the rDNA sequence diversity of a natural population of the arbuscular mycorrhizal fungus *Acaulospora colossica*. Mycorrhiza 13:227–231
Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921
Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales-application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427
Stukenbrock EH, Rosendahl S (2005) Clonal diversity and population genetic structure of arbuscular mycorrhizal fungi (*Glomus* sp.) studied by multilocus genotyping of single spores. Mol Ecol 14:743–752
Suzuki DT, Miller J, Lewontin RC (1986) Introduction to genetic analysis, 3rd edn. Freeman, New York
Trouvelot S, van Tuinen D, Hijri M, Gianinazzi-Pearson V (1999) Visualization of ribosomal DNA loci in spore interphasic nuclei of glomalean fungi by fluorescence *in situ* hyhridization. Mycorrhiza 8:203–206
Vanderkoornhuyse P, Leyval C, Bonnin I (2001) High genetic diversity in arbuscular mycorrhizal fungi: evidence for recombination events. Heredity 87:243–253
Voets L, de la Providencia IE, Declerck S (2006) Glomeraceae and Gigasporaceae differ in their ability to form hyphal networks. New Phytol 172:185–188
Weir BS (1996) Genetic data analysis II: methods for discrete population genetic data, 2nd edn. Sinauer, Sunderland, Mass.
Yilmaz LS, Noguera DR (2004) Mechanistic approach to the problem of hybridization efficiency in fluorescent in situ hybridization. Appl Environ Microbiol 70:7126–7139
Zolan ME (1995) Chromosome-length polymorphism in fungi. Microbiol Rev 59:686–698

Ectomycorrhiza and Water Transport

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1 Introduction

In temperate and boreal regions, seasons are characterized by two major factors – temperature and water availability. As water availability is affecting essential processes like nutrition and photosynthesis, it is of central importance for plant physiology.

Trees and shrubs of temperate and boreal forest ecosystems are characterized by a tight association of their fine roots with certain soil fungi, forming a new symbiotic organ – the ectomycorrhiza. Here, fine roots are often covered by fungal hyphae (the so-called sheath) isolating them from the surrounding soil. Furthermore, fungal hyphae grow within the apoplast of rhizodermis and root cortex, forming a dense hyphal network (Hartig net), which is thought to function as an interface between fungus and plant for the reciprocal exchange of nutrients and metabolites. Mycorrhizas are connected with other parts of the fungal colony (e.g., soil exploring mycelium) by specialized transport hyphae (see below) and, in contrast to a number of well investigated filamentous model ascomycetes (e.g., *Neurospora, Aspergillus*), EM fungal colonies perform intense nutrient and metabolite exchange (for reviews, see Smith and Read 1997; Anderson and Cairney 2007).

In addition to their better nutrition, mycorrhized plants revealed improved water use efficiency (Boyle and Hellenbrand 1990; Loewe et al. 2000; Muhsin and Zwiazek 2002a) and increased CO_2 fixation rates (Friedrich 1998; Wright et al. 2000) in a number of experiments, indicating a lower stomatal resistance and thus an improved water uptake in symbiosis.

Mycorrhiza formation could (1) affect plant water support by introducing additional water transport routes from the soil towards the plant root (the hyphal network), or (2) modulate plants water requisite by improved nutrition and thus reduced transpiration demand.

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2 Impact of Soil Growing Hyphae on Plant Water Support

The common explanation of improved water uptake by mycorrhizal compared to non-mycorrhizal plants (Boyd et al. 1986) is the strongly increased absorbing surface caused by soil growing hyphae combined with the fungal capability to take up water from soils with a lower (more negative) water potential (Mexal and Reid 1973; Theodorou 1978; Theodorou and Bowen 1970; Uhlig 1972).

However, a minimum of about 5,000 fungal species are able to undergo ectomycorrhizal symbiosis with tree and shrub species (Molina et al. 1992), and the ability of soil growing hyphae of EM fungi to support water transport in mycorrhizal association seems to be dependent on their anatomical and physiological properties. Ectomycorrhizal fungi could be grouped by the hydrophobicity of their hyphae (hydrophilic and hydrophobic; Unestam 1991; Unestam and Sun 1995), as well as their strategy in soil exploration (Agerer 2001). While some ECM fungi connect ectomycorrhizas with the soil growing mycelium only by non-specialized hyphae, other form simple or differentiated rhizomorphs (hyphal strands often having enlarged, empty central hyphae surrounded by smaller living hyphae; Agerer 2001; Brownlee et al. 1983). While the hydrophobicity of hyphae could modify water permeability through the fungal sheath and thus the degree of physical isolation of the environed fine root from the surrounding soil, formation of rhizomorphs enables long distance bulk water transport from soil growing hyphae towards ectomycorrhizas and the plant root.

First evidence that water taken up by ECM fungi is transported to the plant was given by Duddridge et al. (1980). By using the same experimental system but providing water only to the physically separated, soil exploring mycelium, Brownlee et al. (1983) demonstrated a directed water transport into the host plant via fungal rhizomorphs. The authors proposed water transport through central, empty vessel hyphae within rhizomorphs, and assimilate transport in the opposite direction by peripheral, cytoplasmatic hyphae. However, in other ectomycorrhizal fungi (without or with less complex rhizomorphs), water transport through the mycelium is managed in an osmotic potential-dependant manner (Duddridge et al. 1980).

3 Ectomycorrhiza Formation Results in Modification of Plant Roots and Affects Their Water Transport Properties

Hydraulic conductance is a measure for the water transport capacity of an organ, e.g., the root system. Comparison of the root hydraulic conductance of mycorrhized and non-mycorrhized tree root systems provided an insight into this subject (Nardini et al. 2000; Muhsin and Zwiazek 2002a, 2002b; Landhauser et al. 2002) and revealed ecologically dependent plant adaptations.

Root hydraulic conductance could be affected by two mechanisms: (1) changes in root hydraulic conductivity (Lp), a measure for apoplastic and symplastic water

transport properties caused by changes in cell wall composition and plasma membrane water permeability, and (2) modulation of the whole root system (e.g., modification of its size or the number of fine roots).

Evergreen oak (*Qercus ilex*) seedlings that were mycorrhized with *Tuber melanosporum* revealed a 2.5-times lower root hydraulic conductivity than non-inoculated control plants, indicating a lower water transport capacity of infected fine roots. As, at the whole plant level, inoculated evergreen oak seedlings showed better performances (Nardini et al. 2000), additional adaptations of mycorrhized plants (e.g., better water use efficiency, improved nutrition) that are able to overcompensate the reduced hydraulic conductivity of the root system in symbiosis could be supposed. Furthermore, in Mediterranean environments, where both symbionts are native and which are characterized by seasonal water shortage, reduced hydraulic conductivity could help to survive drought periods, as not only water uptake but also potential water loss is reduced. Similar to Mediterranean Evergreen oak, a reduction in Lp was also observed for Norway spruce (Marjanovic 2004; Marjanovic et al., unpublished), a tree that is characterized by its adaptation to winter drought (Kottke et al. 1997).

On the other hand, root hydraulic conductivity was increased upon ectomycorrhiza formation in *Ulmus americana, Picea glauca* and *Populus tremuloides*, temperate to boreal trees often living in the colder, water saturated soils. A tight control of potential water loss seems thus to be of a special importance for trees adapted to conditions of regular water shortage.

Increased root hydraulic conductivity in *U. americana* (Muhsin and Zwiazek 2002a, 2002b) and *P. tremula* x *tremuloides* in symbiosis (Marjanovic et al. 2005b) is, however, accompanied by the formation of smaller root systems. In contrast, the root system of Norway spruce (revealing reduced root hydraulic conductivity) increased in size upon ectomycorrhiza formation. It could thus be speculated that plants may have two different strategies to react upon ectomycorrhiza formation: decrease of root system size accompanied with increased root hydraulic conductivity and visa versa.

4 Aquaporins: Gates for Symplastic Water Transport

In roots, radial water transport is a combination of apoplastic, symplastic, and transcellular pathways (Steudle and Peterson 1998). Which of them will be prevailing depends on the current situation, e.g., plant nutritional and water status, cell wall chemistry and environmental factors (Steudle and Peterson 1998; Schreiber et al. 2005). However, water has to pass at least at certain points (exo- and endodermal barriers, e.g., Casparian band, which presumably functions as a check point for water uptake and loss) the plasma membrane before entering the vascular system.

At the time of the first experiments on ectomycorrhiza connected water transport, the dogma of free diffusion through membranes was widely accepted as the common way of water transport. The surprise came at the beginning of 1990s when

Preston et al. (1992) discovered a new family of membrane transporters whose presumably primary function was to facilitate water transport driven by the osmotic gradient inside and outside the cell – the aquaporins (AQP; Chrispeels and Agre 1994). Sequencing of *Arabidopsis thaliana* (Johanson et al. 2001) and *Zea mays* (Chaumont et al. 2001) genomes revealed large AQP families in plants containing around 35 different members that could be divided into four subfamilies according to their localization (Chaumont et al. 2005; Ishikawa et al. 2005): plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin like intrinsic proteins (NIPs, localized in the perisymbiotic membrane of legumes), and small intrinsic proteins (SIPs, present in the endoplasmatic reticulum and/or some other inner membranes). Due to the ubiquitous presence of aquaporins in plant membranes and the ability of plants to restrict water flux through them by post-translational modification (Luu and Maurel 2005; Kaldenhoff and Fischer 2006), plants could perhaps tightly control membrane water permeability.

However, in addition to water, some AQPs are able to transport small uncharged molecules like glycerol, urea or CO_2 (Biela et al. 1999; Gerbeau et al. 2002; Uehlein et al. 2003; Weig and Jakob, 2000), and presence of AQPs in certain membranes might thus occur due to other reasons than water transport.

5 Modulation of Plasma Membrane Water Transport Capacity in Poplar Fine Roots by Ectomycorrhiza Formation

Aquaporin content in plant membranes is tightly regulated at the level of gene expression. Environmental (water, energy and nutrient status) and intrinsic (development, cytoplasmic pH, hormones) factors do influence AQP expression (Guerrero et al. 1990; Yamaguchi-Shinozaki et al. 1992; Azaizeh et al. 1992; Kaldenhoff et al. 1993; Phillips and Huttly 1994; Yamada et al. 1995; Maurel and Chrispeels 2001; for review, see Chaumont et al. 2005).

As, in poplar, both apoplastic and symplastic water transport activity are affected upon ectomycorrhiza formation (Marjanovic et al. 2005a), the impact of the symbiosis on aquaporin gene expression is of special interest.

Kohler et al. (2003) identified a total of six PIPs (three members of PIP1 and PIP2 subgroup each) and five TIP genes in a large scale EST sequencing project of poplar root cDNA libraries. All members of the PIP1 subgroup were repressed upon poplar fine root formation while the expression of two members of the PIP2 subgroup was enhanced (Kohler et al. 2003).

Using degenerated primers, eight potential AQP genes (seven PIPs and one SIP) could be amplified from first strand cDNA of fully developed *Populus tremula* x *tremuloides/Amanita muscaria* ectomycorrhizas (Marjanovic et al. 2005b), revealing good homology (but not identity) with ESTs obtained from *P. trichocarpa x deltoides* by Kohler et al. (2003). As the *Populus trichocarpa* genome contains a total of five members of the PIP1 and nine members of the PIP2 subgroup (Fig. 1, Tuskan et al. 2006), the seven PIP genes (two members of PIP1, five members of PIP2)

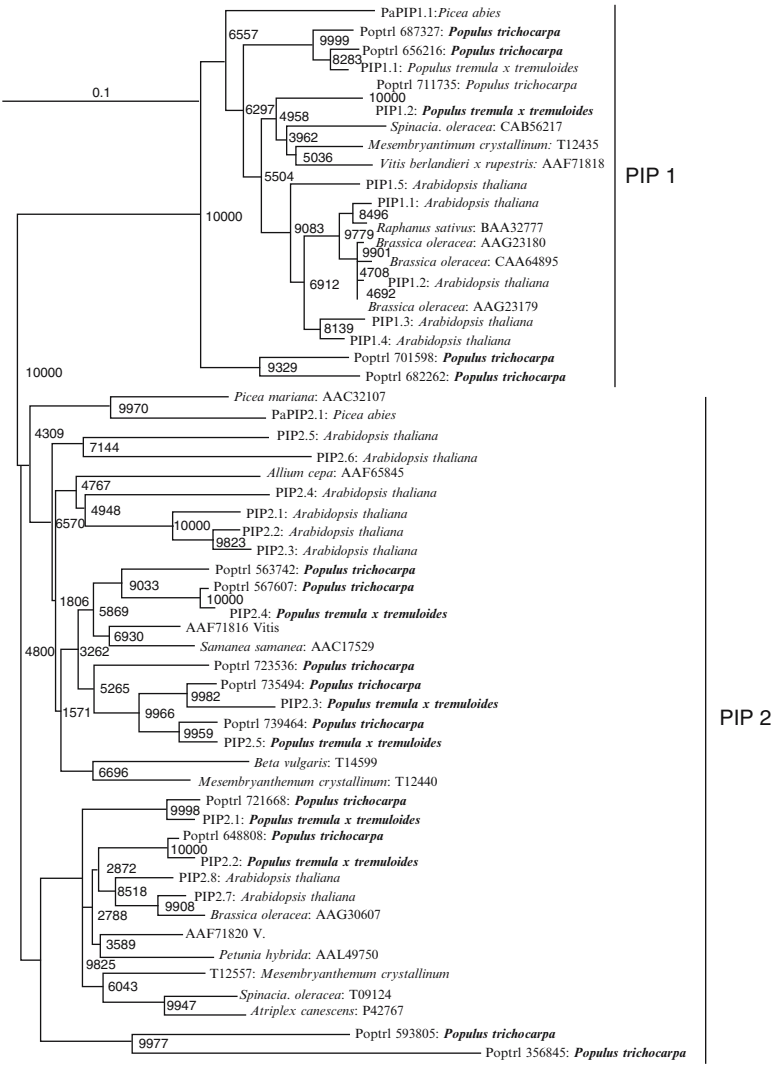

Fig. 1 Dendrogram of deduced PIP proteins from genomic DNA sequences of *Populus trichocarpa* (Tuskan et al. 2006) and *Arabidopsis thaliana*, and cDNAs of *P. tremula* x *tremuloides* (Marjanovic et al. 2005b) and other selected plants. Dendrogram and bootstrap values were calculated using ClustalX and visualized using TreeView (http://taxonomy.zoology.gla. ac.uk/rod/treeview.html). Gene names (if available) or accession numbers are shown together with species names

characterized by Marjanovic et al. (2005b) represent about half of the poplar PIPs. Interestingly, no TIP encoding gene was amplified in this study even when the primers were designed to amplify all AQP subgroups, presumably indicating that TIP transcripts might be present at a significantly lower levels compared to those of PIP genes upon ectomycorrhiza formation. As the expression of four out of the seven

identified poplar PIP genes was enhanced in ectomycorrhizas, increased water transport capacity could be expected in the plasma membrane of mycorrhized poplar fine roots (Marjanovic et al. 2005b). This is in agreement with the observed increase in root hydraulic conductance (Marjanovic et al. 2005a), indicating enhanced water transport capacities for both the cell wall and the plasma membrane. Root-located expression of the identified poplar SIP gene in mycorrhized plants (Marjanovic 2004) may indicate domination of the root symplastic water transport pathway upon symbiosis establishment. SIPs with high sequence similarity to poplar PttSIP1.1 were localized in the endoplasmatic reticulum of *Arabidopsis* roots (Ishikawa et al. 2005).

One of the identified poplar PIPs (PttPIP2.5) is of a special interest since transcripts of the respective gene were highly abundant in nonmycorrhized fine roots and further enhanced upon ectomycorrhiza formation. As the corresponding protein revealed a strong water transport activity when heterologously expressed in *Xenopus laevis* oocytes, Marjanovic et al. (2005b) supposed that PttPIP2.5 might be responsible for the observed increase in plasma membrane permeability of poplar fine roots in symbiosis. Furthermore, the expression of this gene was also affected under drought conditions. While transcript levels remained unchanged in mycorrhized plants, they strongly decreased in fine roots of non-mycorrhized plants (Marjanovic et al. 2005b), indicating a stabilizing effect of mycorrhizas on plant physiology during environmental changes.

In agreement with data obtained from poplar, the expression of an aquaporin gene was also induced during fine root and ectomycorrhiza formation in oak (Frettinger et al. 2007). In contrast, investigations with *Betula pendula* (Johansson et al. 2004; Le Quere et al. 2005) and Norway spruce (Marjanovic et al., unpublished) revealed the repression of certain aquaporin genes in ectomycorrhizas. However, as a number of PIP genes are transcribed in tree roots, expression behavior of individual aquaporin genes is difficult to interpret without further functional information.

6 Impacts of Ectomycorrhizas on Trees under Drought Conditions

Early experiments conducted on the impact of ectomycorrhizas on plant behavior under drought conditions concluded that this symbiosis could have positive, neutral, or even negative effects on plant growth (Sands and Teodorou 1978; Dixon et al. 1980; Sands et al. 1982; Parke et al. 1983; Coleman et al. 1990; Lamhamedi et al. 1992). The situation became clearer when Lehto (1992a, 1992b) demonstrated the importance of taking into consideration the plant nutritional status. Under nutrient limited and water saturated conditions, establishment of ectomycorrhiza between *Picea sitchensis* and *Paxillus involutus* improved plant performance. However, when mycorrhized trees were brought to a similar nutrient status as non-mycorrhizal plants, growth improvement in symbiosis was no longer significant. It was thus concluded that improved plant performance in symbiosis is mainly the result of

better nutrition. Whether mycorrhizal symbiosis is useful for plant growth under drought is accordingly dependent on whether an EM fungus is capable to obtain nutrients from soil and transfer them towards the plant under these conditions.

As fungal hyphae are significantly smaller than even root hairs and the mycelium has a 100- to 1,000-times larger surface area, immobile nutrients are taken up with much higher efficiency from soil particles and are transferred via the hyphal network over long distances (Boyd et al. 1986; Rousseau et al. 1994; Bending and Read 1995; Perez-Moreno and Read 2000). Thus, the need for bulk water transport for mineral nutrition is reduced/avoided (dependent on fungal transport mechanisms) and plants could obtain similar amounts of nutrients at a reduced water demand.

If the soil moisture is too low and the fungus is hardly surviving itself, no beneficial effect for the host plant will be observed (Kennedy and Peay 2007). However, the level of drought sensitivity of an EM fungus is depending on its physiological capacity. Under drought conditions, *Coenococcum geophylum* mycorrhizas keep water bound to gelatinous structures and start functioning instantly after re-watering (Pigott 1982; di Pietro et al. 2007; Agerer 2001; Jany et al. 2003), indicating that certain EM fungi are adopted to sustain drought/re-watering cycles and revitalize quite fast thereafter. As a consequence, these fungi are able to support plant nutrition soon after a desiccation period. Accordingly, in sandy or stony acidic soils where periodical drought conditions are common, *Coenococcum*-like EM fungi were found to be dominating mycorrhizal tips of *Tilia* sp. (Pigott 1982).

Queretejeta et al. (2003) brought a further aspect of plant–fungus relationship in ectomycorrhizal symbiosis into focus that could help both partners to survive under drought conditions, the so called “hydraulic lift”. In the night, when plant transpiration is strongly reduced (Dawson 1993; Caldwell et al. 1998; Horton and Hart 1998), water is transported by tap roots from lower, water-containing regions via fine roots and the mycorrhizal network into upper soil layers. The driving force for this transport is the water potential difference between lower and upper soil compartments and lack of a transpiration stream. Water transport in ectomycorrhizas could therefore occur in both directions, depending on which partner is in position to absorb it (Queretejeta et al. 2003).

7 Conclusions

Ectomycorrhiza formation could influence host plant water balance at different levels: (1) modulation of water use efficiency (improved nutrition at lower water demand), (2) modification of the entire root system (by modulation of fine root formation), and (3) modulation of root hydraulic conductivity (by modification of cell wall properties and membrane permeability). Which level will be more affected and to what extent is dependant on the genetic determination of the involved partners and could explain, together with soil properties, the variance of plant responses observed in symbiosis.

References

Agerer, R (2001) Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11:107–114

Anderson IC, Cairney JWG. 2007. Ectomycorrhizal fungi: exploring the mycelial frontier. FEMS Microbiol Rev 31:388–406

Azaizeh H, Gunse B, Steudle E (1992) Effects of NaCl and $CaCl_2$ on water transport across root cells of maize (*Zea mays* L.) seedlings. Plant Physiol 99:886–894

Bending GD & Read DJ. (1995) The structure and function of the vegetative mycelium of ectomycorrhizal plants V. Foraging behaviour and translocation of nutrients from exploited litter. New Phytol 130:401–409

Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R (1999) The Nicotiana tabacum plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. Plant J 18:565–570

Boyd R, Furbank RT, Read DJ (1986) Ectomycorrhiza and the water relations of trees. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 689–693

Boyle CD, Hellenbrand KE (1990) Assesment of the effect of mycorrhizal fungi on drought tolerance of conifer seedlings. Can J Bot 69:1764–1771

Brownlee C, Duddridge JA, Malibari A, Read DJ (1983) The structure and function of mycelial systems of ectomycorrhizal roots with special reference to their role in forming inter-plant connections and providing pathways for assimilate and water transport. Plant Soil 71:433–443

Caldwell MM, Dawson TE, Richards JH (1998) Hydraulic lift: consequences of water efflux from the roots of plants. Oecologia 113:151–161

Chaumont F, Barrieu F, Wojcik E, Chrispeels MJ, Jung R (2001) Aquaporins constitute a large and highly divergent protein family in maize. Plant Physiol 125:1206–1215

Chaumont F, Moshelion M, Daniels MJ (2005) Regulation of plant aquaporin activity. Biol Cell 97:749–64

Chrispeels MJ, Agre P (1994) Aquaporins: Water channel proteins of plant and animal cells. Trends in Biochemical Sciences 19(10):421–425

Coleman MD, Bledsoe CS, Smit BA (1990) Root hydraulic conductivity and xylem sap levels of zeatin riboside and abscisic acid in ectomycorrhizal Douglas fir seedlings. New Phytol 115:275–284

Dawson TE (1993) Hydraulic lift and water use by plants: implications for water balance, performance and plant-plant interactions. Oecologia 95:565–574

Dixon RK, Wright GM, Behrns GT, Teskey RO, Hinckley TM (1980) Water deficits and root growth of ectomycorrhizal white oak seedlings. Can J For Res 10:545–548

Duddridge JA, Malibari A, Read DJ (1980) Structure and function of mycorrhizal rhizomorphs with special reference to their role in water transport. Nature 287:834–835

Frettinger, P, Derory J, Herrmann S, Plomion C, Lapeyrie F, Oelmuller R, Martin F, Buscot F (2007) Transcriptional changes in two types of pre-mycorrhizal roots and in ectomycorrhizas of oak microcuttings inoculated with *Piloderma croceum*. Planta 225:331–40

Friedrich A (1998) Regulation des Kohlenhydratstoffwechsels in Blättern mykorrhizierter Fichten (*Picea abies* [L.] Karst.) und Zitterpappeln (*Populus tremula x tremuloides* Mich.) unter erhöhter CO2-Konzentration. Thesis, Physiologische Ökologie der Pflanzen, Tübingen

Gerbeau P, Amodeo G, Henzler T, Santoni V, Ripoche P, Maurel C (2002) The water permeability of Arabidopsis plasma membrane is regulated by divalent cations and pH. Plant J 30:71–81

Guerrero FD, Jones JT, Mullet JE (1990) Turgor-responsive gene transcription and RNA levels increase rapidly when pea shoots are wilted. Sequence and expression of three inducible genes. Plant Mol Biol 15:11–26

Horton JL, Hart SC (1998) Hydraulic lift: a potentially important ecosystem process. Trends Ecol Evol 13:232–235

Ishikawa F, Suga S, Uemura T, Sato MH, Maeshima M (2005) Novel type aquaporin SIPs are mainly localized to the ER membrane and show cell-specific expression in *Arabidopsis thaliana*. FEBS Lett 579:5814–5820

Jany JL, Bousquet J, Khasa DP (2003) Microsatellite markers for *Hebeloma* species developed from expressed sequence tags in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol Ecol Notes 3:659–661

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjöval S, Fraysse L, Weig AR, Kjellbom P (2001) The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 126:1358–1369

Johansson T, Le Quere A, Ahren D, Söderström B, Erlandsson R, Lundeberg J, Uhlen M, Tunlid A (2004) Transcriptional responses of *Paxillus involutus* and *Betula pendula* during formation of ectomycorrhizal root tissue. Mol Plant Microb Interact 17:202–215

Kaldenhoff R, Fischer M (2006) Functional aquaporin diversity in plants. Biochim Biophys Acta 1758:1134–1141

Kaldenhoff R, Kolling A, Richter G (1993) A novel blue light- and abscisic acid-inducible gene of *Arabidopsis thaliana* encoding an intrinsic membrane protein. Plant Mol Biol 23:1187–1198

Kennedy PG, Peay KG (2007) Different soil moisture conditions change the outcome of the ectomycorrhizal symbiosis between *Rhizopogon* species and *Pinus muricata*. Plant Soil 291:155–165

Kohler A, Delaruelle C, Martin D, Encelot N, Martin F (2003) The poplar root transcriptome: analysis of 7000 expressed sequence tags. FEBS Lett 542:37–41

Kottke I, Münzenberger B, Oberwinkler F (1997) Structural approach to function in ectomycorrhizae. In: Rennenberg H, Eschrich W, Ziegler H (eds) Trees – contribution to modern tree physiology. Backhuys, Leiden, pp 357–376

Lamhamedi MS, Bernier PY, Fortin JA (1992) Hydraulic conductance and soil water potential at the soil root interface of *Pinus pinaster* seedlings inoculated with different dikaryons of *Pisolithus* sp. Tree Physiol 10:231–244

Landhäusser SM, Muhsin TM, Zwiazek JJ (2002) The effect of ectomycorrhizae on water relations in aspen (*Populus tremuloides*) and white spruce (*Picea glauca*) at low soil temperatures. Can J Bot 80:684–689

Le Quere A, Wright D, Söderström B, Tunlid A (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) Fr. Mol Plant Microb Interact 18:659–673

Lehto T (1992a) Mycorrhiza and drought resistance of *Picea sitchensis* (Bong.) carr. I. In conditions of nutrient deficiency. New Phytol 122:661–668

Lehto T (1992b) Mycorrhizas and drought resistance of *Picea sitchensis* (Bong.) carr. II. In conditions of adequate nutrition. New Phytol 122:669–673

Loewe A, Einig W et al. (2000) Mycorrhiza formation and elevated CO2 both increase the capacity for sucrose synthesis in source leaves of spruce and aspen. New Phytol 145:565–574

Luu D-T, Maurel C (2005) Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ 28:85–96.

Marjanovic Ž (2004) Impact of mycorrhiza formation and drought stress on the expression and function of aquaporins in Norway spruce (*Picea abies* (L.) Karst.) and hybrid aspen (*Populus tremula* L. x *Populus tremuloides* Mich.) PhD Thesis, Karl-Eberhard Universität Tübingen, Germany

Marjanovic Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weia M, Hampp R, Nehls U (2005a) Aquaporins in poplar: what a difference a symbiont makes! Planta 222:258–268

Marjanovic Z, Nehls U, Hampp R (2005b) Mycorrhiza formation enhances adaptive response of hybrid poplar to drought. Ann NY Acad Sci 1048:496–499

Maurel C, Chrispeels JM (2001) Aquaporins – a molecular entry into plant water relations. Plant Physiol 125:135–138

Mexal J, Reid CPP (1973) The growth of selected mycorrhizal fungi in response to induced water stress. Can J Bot 51:1579–1588

Molina R, Massicotte H, Trappe JM (1992) Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen MF (ed) Mycorrhizal functioning: an integrative plant–fungal process. Chapman and Hall, New York, pp 357– 423

Muhsin TM, Zwiazek JJ (2002a) Ectomycorrhizas increase apoplastic water transport and root hydraulic conductivity in *Ulmus americana* seedlings. New Phytol 153:153–158

Muhsin TM, Zwiazek JJ (2002b) Colonization with *Hebeloma crustuliniforme* increases water conductance and limits shoot sodium uptake in white spruce (*Picea glauca*) seedlings. Plant Soil 238:217–225

Nardini A, Salleo S, Lo Gullo MA, Pitt F (2000) Different responses to drought and freeze stress of *Quercus ilex* L. growing along a latitudinal gradient. Plant Ecol 148:139–147

Parke JL, Linderman RG, Black CH (1983) The role of mycorrhizas in drought tolerance of Douglas-fir seedlings. New Phytol 95:83–95

Perez-Moreno J, Read DJ (2000) Mobilization and transfer of nutrients from litter to tree seedlings via the vegetative mycelium of ectomycorrhizal plants. New Phytol 145:301–309

di Pietro M, Churin JL, Garbaye J (2007) Differential ability of ectomycorrhizas to survive drying. Mycorrhiza (in press)

Phillips AL, Huttly AK (1994) Cloning of two gibberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: expression of the tonoplast water channel, TIP, is increased by GA 3. Plant Mol Biol 24:603–615

Pigott CD (1982) Survival of mycorrhiza formed by *Cenococcum geophilum* Fr. in dry soils. New Phytol 92:513–517

Preston GM, Carroll TP, Guggino WB, Agre P (1992) Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science 256:385–387

Queretejeta JF, Egerton-Warburton LI, Allen MI (2003) direct nocturnal water transfer from oaks to their mycorrhizal symbionts during severe soil drying. Oecologia 134:55–64

Rousseau BVD, Sylvia DM, Fox AJ (1994) Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. New Phytol 128:639–644

Sands R, Theodorou C (1978) Water uptake by mycorrhizal roots of Radiata pine seedlings. Aust J Plant Physiol 5:301–309

Sands R, Fiscus EL, Reid CPP (1982) Hydraulic properties of pine and bean roots with varying degrees of suberization, vascular differentiation and mycorrhizal infection. Aust J Plant Physiol 9:559–569

Schreiber L, Franke R, Hartmann K-D, Ranathunge K, Steudle E (2005). The chemical composition of suberin in apoplastic barriers affects radial hydraulic conductivity differently in the roots of rice (*Oryza sativa* L. cv. IR64) and corn (*Zea mays* L. cv. Helix) J Exp Bot 56:1427–1436

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, San Diego, Calif.

Steudle E, Peterson CA (1998) How does water get through roots? J Exp Bot 49:775–788

Theodorou C (1978) Soil moisture and the mycorrhizal association of *Pinus radiata* D. Don. Soil Biol Biochem 10:33–37

Theodorou C, Bowen GD (1970) Mycorrhizal responses of radiata pine in experiments with different fungi. Aust For 34:183–191

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313:1596–1604

Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R (2003) The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. Nature 425:734–737

Uhlig SK (1972) Untersuhungen zur Trockenresistenz mycorrhizabildender Pilze. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg B 127:124–133

Unestam T (1991) Water repellency, mat formation and leaf-stimulated growth of some ectomycorrhizal fungi. Mycorrhiza 1:13–20

Unestam T, Sun YP (1995) Extramatrical structures of hydrophobic and hydrophilic ectomycorrhizal fungi. Mycorrhiza 5:301–311

Weig AR, Jakob C (2000) Functional identification of the glycerol permease activity of *Arabidopsis thaliana* NLM1 and NLM2 proteins by heterologous expression in Saccharomyces cerevisiae. FEBS Lett 481:293–298

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in *Betula pendula* Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. Plant Cell Environ 23:39–49

Yamada S, Katsuhara M, Kelly WB, Michalovski CB, Bohnert HJ (1995) A family of transcripts encoding water channel proteins: tissue-specific expression in the common ice plant. Plant Cell 7:1129–1142

Yamaguchi-Shinozaki K, Koizumi M, Urao S, Shinozaki K (1992) Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana:* sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol 33:217–224

Hypogeous Pezizaceae: Physiology and Molecular Genetics

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1 Introduction

With the exception of the forest truffles, which belong to the Tuberaceae family, truffle members of the order Pezizales may be said to have been rather neglected by science. The limited research that has been done on non-Tuberacean hypogeous mushrooms of the Pezizales, which include the desert truffles, has mostly focused on Phylogenetic aspects. Traditionally, the assignment of a particular family and species of the truffle genera to a particular genus was based on morphological parameters of the ascomata, asci and spores (Trappe 1979). In recent years, however, molecular Phylogenetic research on sequestrate fungi has repeatedly demonstrated that the morphology of hypogeous fungi can be misleading due to reduction in macromorphological characters needed for distinguishing their related epigeaous taxa. Molecular analyses of members of the Pezizales and of the Phylogenetic relations among epi- and hypogeous species have been conducted by O'Donnell et al. (1997), Norman and Egger (1999), Percudani et al. (1999), Roux et al. (1999), Hansen et al. (2001) and Diez et al. (2002). Some of these investigations validate earlier morphological findings. Thus, O'Donnell et al. (1997) and Hansen et al. (2001) demonstrated that certain hypogeous members of the Pezizales show greater affinity to epigeous members than to other hypogeous species. Similarly, the monophyletic origin of some members of *Terfezia* and *Tirmania* was substantiated by Diez et al. (2002). However, other results have upset some of the earlier placements. For example, along with *Tirmania*, *Terfezia* was shown to belong to the Pezizaceae rather than to the distinctly hypogeous Terfeziaceae family, which was therefore abolished (Norman and Egger 1999; Percudani et al. 1999). The genus *Choiromyces* was transferred from the Pezizaceae (Terfeziaceae) to the Tuberaceae (O'Donnell et al. 1997; Percudani et al. 1999), although one *Choiromyces* species, *C. echinulatus*, was excised from this genus and restored to the Pezizaceae under a new name, *Eremiomyces echinulatus* (Ferdman et al. 2005). Similarly, two species

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were removed from the *Terfezia* genus: *Terfezia terfezioides*, reinstated as *Mattirolomyces terfezioides* (Percudani et al. 1999; Diez et al. 2002), and *Terfezia pfeilii*, renamed *Kalaharituber pfeilii* (Ferdman et al. 2005). Cryptic species are coming to light, including a group of three discovered in the *T. boudieri* desert truffle complex (Ferdman 2006). Moreover, new mycorrhizal species were recently identified among the Pezizales (Tedersoo et al. 2006).

The volume of research undertaken on the physiology, biochemistry, and plant–fungus relations of the hypogeous members of Pezizaceae is even smaller, with the result that our knowledge about these topics is fragmentary.

Less than exhaustive studies of the composition and nutritional value of desert truffles have been carried out in those countries where they are known and appreciated. It appears that the dry matter (about 20% by weight) consists of: 20–27% protein, some 85% of which is digestible by humans; 3–7.5% fat, including unsaturated as well as saturated fatty acids; 7–13% crude fiber; close to 60% carbohydrates; and appreciable amounts (2–5%) of ascorbic acid (Ackerman et al. 1975; Al-Delaimy 1977; Al-Shabibi et al. 1982; Sawaya et al. 1985; Sakri 1989; Bokhari et al. 1989; Bokhari and Parvez 1993; Murcia et al. 2003). High levels of potassium and phosphate and fair amounts of iron have been reported (Sakri 1989). No known and tested toxic plant compound has been detected (Ahmed-Ashour et al. 1981).

The recent spurt of interest in desert truffles as potential candidates for cultivation (Honrubia et al. 2002; Kagan-Zur et al. 1999) will hopefully motivate more probing investigation of the genera involved and has already generated several (albeit limited) biochemical studies of enzymatic activities in one species of desert truffle (Perez-Gilabert et al. 2001a, 2001b, 2004, 2005; and see *Biotechnology and Cultivation of Desert Truffles* in this volume). In this chapter, we will review what else is known about these truffles and what can be inferred from initial results.

2 Unresolved Problems in the Life Cycle of Underground Pezizaceae

There are fundamental aspects in the biology of underground Pezizaceae in particular and truffles in general that have yet to be fully elucidated, including the mating system and the ploidy level of both mycorrhizal and sterile fruit-body hyphae. Both are the subject of conflicting hypotheses still awaiting resolution.

Tuber and *Terfezia* hyphal cells, like those of other Ascomycetes, are multinucleate, so ascertaining ploidy level is problematic. Nowadays, however, the prevailing perception, which is based on in-depth research, casts doubt on the long-term subsistence of natural vegetative heterokaryons in filamentous Ascomycetes (Glass et al. 2000).

The hypothesis that tends to be accepted today – a hypothesis that has been thoroughly investigated – maintains that the vegetative mycelium of such fungi is homokaryotic and that plasmogamy occurs only in the primordia of the fruit bodies. Plasmogamy in the developing fruit body leads to the formation of heterokaryotic

ascogenous hyphae, from which, after karyogamy and meiosis, haploid ascospores develop (Alexopoulos et al. 1996). As a result, the heterokaryotic phase is brief and is restricted to the developing ascocarps.

Yet it has consistently been assumed that the ascocarps of *Tuber melanosporum* and *T. magnatum* – the black Perigord truffle and the white Piedmont truffle, respectively – are diploid (heterokaryotic), and they have therefore been regarded as selfing species. The reason is that when codominant markers were evaluated, heterozygous ascocarps were not detected (Bertault et al.1998, 2001; Frizzi et al. 2001; Mello et al. 2005; Murat et al. 2003). Also, the results of an old experiment by Bonfante-Fasolo and Brunell (1972) suggested that monosporic mycelia could not mycorrhize while multisporic cultures could.

Using microsatellite markers, Paolocci et al. (2006) demonstrated that some and possibly considerable outcrossing takes place in *Tuber magnatum*. According to the authors, the vegetative infecting mycelium is homokaryotic, originating from single ascospore germination, and, in line with current dogma, they maintain that fertilization occurs in the primordia of the ascocarps. They also maintain that the donor of the genetic complement could be asexual spores (conidia), which have recently been detected in *Tuber borchi* and *T. oligospermum* (Urban et al. 2004). The ascogenous heterokaryotic hyphae resulting from the fertilization process are surrounded by homokaryotic sterile maternal vegetative hyphae, and the mature ascocarps are therefore composed exclusively of asci and homokaryotic gleba and sterile veins. Of course, since as many as 75% of the supposedly single-spore mycorrhizal root tips were of purely maternal origin (the expected outcome was 25%), considerable selfing probably occurred. The finding that single root tips harbored only one combination of marker alleles is certainly compelling, though the use of microsatellites as markers could be viewed as problematic owing to the possibility of instability, especially during meiosis.

In any event, findings for *Terfezia boudieri* and *Kalaharituber pfeilii* cannot easily be reconciled with the chain of events described by Paolocci et al. (2006):

1. Though different culture media were tried, our experience was that singly germinated spores of *T. boudieri* fail to develop a viable mycelium and perish at an early stage (unpublished results). Germinated single spores of *K. pfeilii* do develop on such media, but their mycelial culture viability seems to dwindle away after a few rounds of sub culturing. In our hands, long-lived *T. boudieri* and *K. pfeilii* cultures were invariably derived from glebal outgrowth (sterile hyphae) or multi-spore germination (*K. pfeilii*); these mycelia were successfully used in mycorrhization experiments (Roth-Bejerano et al. 1990; Kagan-Zur et al. 1994).
2. In the above-mentioned long-lived *K. pfeilii* and *T. boudieri* cultures, the hyphal cells invariably contained paired nuclei (Roth-Bejerano et al. 2004) (Figs. 1 and 2). *K. pfeilii* hyphae originating from single ascospore cultures showed unpaired nuclei, while hyphae obtained at the contact zone between two different single-spore-derived mycelia had paired nuclei similar to gleba derived cultures (ibid). Similar findings have been reported for *Morchella* (Volk and Leonard 1990).

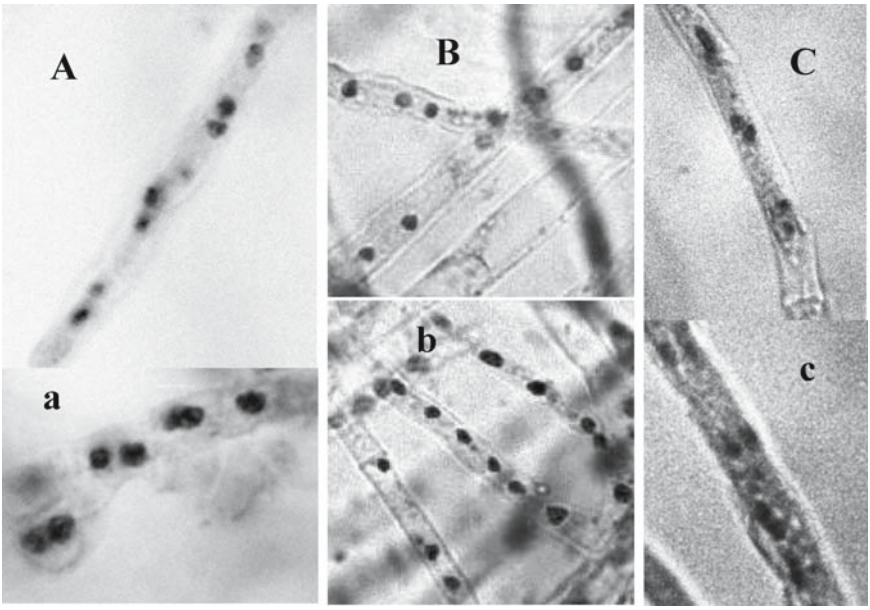

Fig. 1 Hyphae of *Kalaharituber pfeilii* containing multinucleate cells: *A,a* hyphae originating from sterile fruit-body veins; *B,b* hyphae originating from a single germinating spore; *C,c* hyphae obtained at the contact zone between two homokaryotic mycelia, each of which originated from a single ascospore. Magnification X 1,000. From Roth-Bejerano et al. (2004), with kind permission of Springer Science and Business Media

3. One such paired-nuclei culture actually contained two different variants of the ITS (Internal Transcribed Spacer of the rRNA gene cluster) region (Fig. 3) in a single hypha. This culture has now been maintained for about 10 years without any alteration taking place in the (equal) intensity of the two variants. Had the two ITS types been contained together within each nucleus in the culture, then according to the theory of molecular drive processes and concerted evolution (Dover 1982, 1986), one should at least have begun to overtake the other. Ganley and Scott (1998) have demonstrated the rapid appearance (within two generations of single spore purification) of a range of ribosomal spacer heterogeneity following karyogamy in a hybrid fungal endophyte where each parent had a single and stable form of spacer; their conclusion was that unification of new forms may take place within a few years. The fact that such unification did not occur in the case of our *T. boudieri* ITS variants seems to support the possibility of long-term maintenance of the two forms in separate nuclei, i.e. heterokaryotic culture (Aviram et al. 2004).
4. Two fruit bodies of *K. pfeilii* were found to harbor two ITS forms each. As no glebal culture could be obtained from the dry fruit bodies, it is not clear whether the two were maintained in intertwined separate hyphae or, as in the double-profile

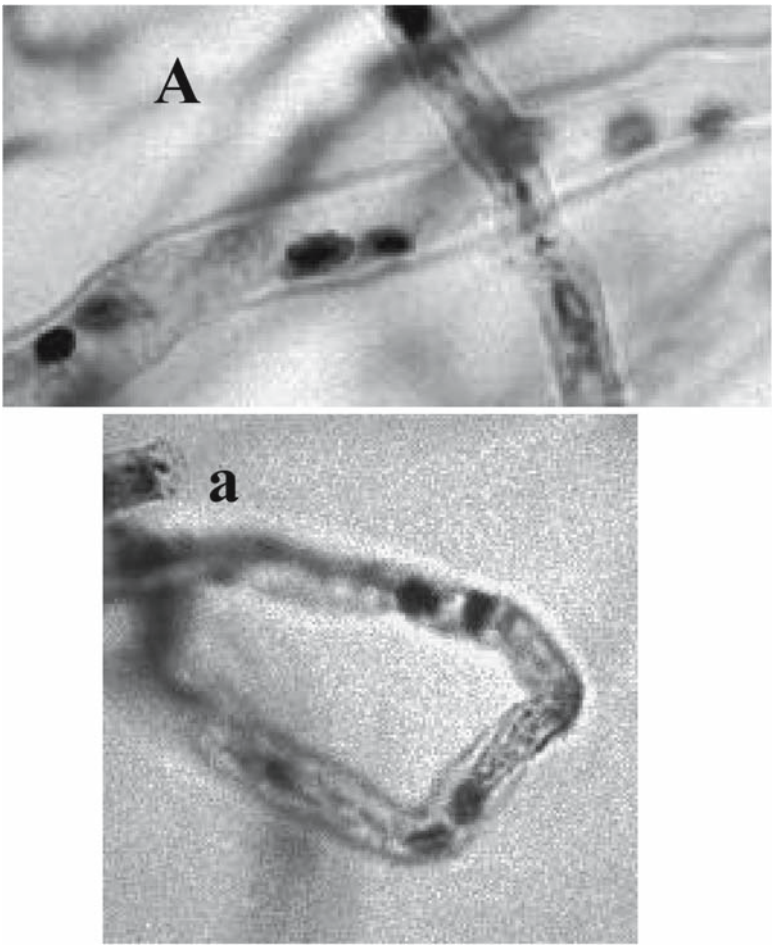

Fig. 2 Hyphae from a *Terfezia boudieri* culture developed from sterile glebal veins. Multinucleate cells with paired nuclei are clearly seen. Magnification × 1,000. From Roth-Bejerano et al. (2004), with kind permission of Springer Science and Business Media

culture described above, in separate nuclei sharing the same cytoplasm. Each germinated ascospore harbored only one of the two forms (Kagan-Zur et al. 1999). The mere existence of such heterozygous fruit bodies seems to contradict the notion of a non-hyphal genetic complement donor.

Do the Tuberaceae and the Pezizaceae differ in their life cycle? Is their position at opposite ends of the pezizalean tree (Norman and Egger 1999; Percudani et al. 1999), distant enough? For at least the underground Pezizaceae and possibly other genera or families, a likely scenario consistent with the evidence would include the following stages (Fig. 4). Ascospores germinate to produce the primary homokaryotic mycelium. This mycelium develops in the soil, where it either inoculates host

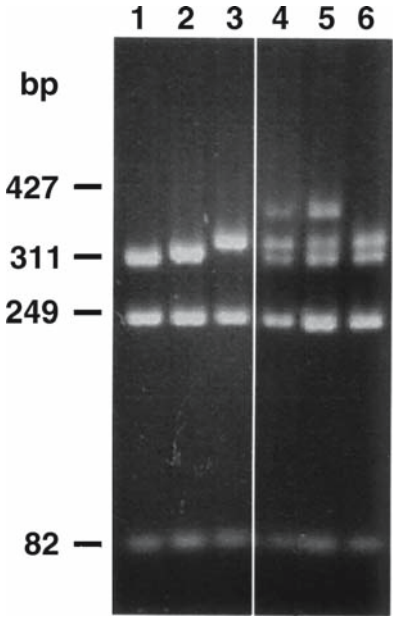

Fig. 3 Comparison of *Hinf*I restricted *ITS*-RFLP profiles of two forms co-existing in a mycelium originating from a section of a single hypha (culture 42a). Lane *1*: cloned 42a(i) (shorter sequence, never encountered before); lane *2*: form 1; lane *3*: form 2 (cloned 42a(ii), the longer sequence); lane *4*: original, not cloned, 42a; lane *5*: mixed and denatured 42a(i) and 42a(ii); lane *6*: a non-denatured mixture of 42a(i) and 42a(ii) proving that the higher band is a heteroduplex of the two forms. From Aviram et al. (2004), with kind permission of Springer Science and Business Media

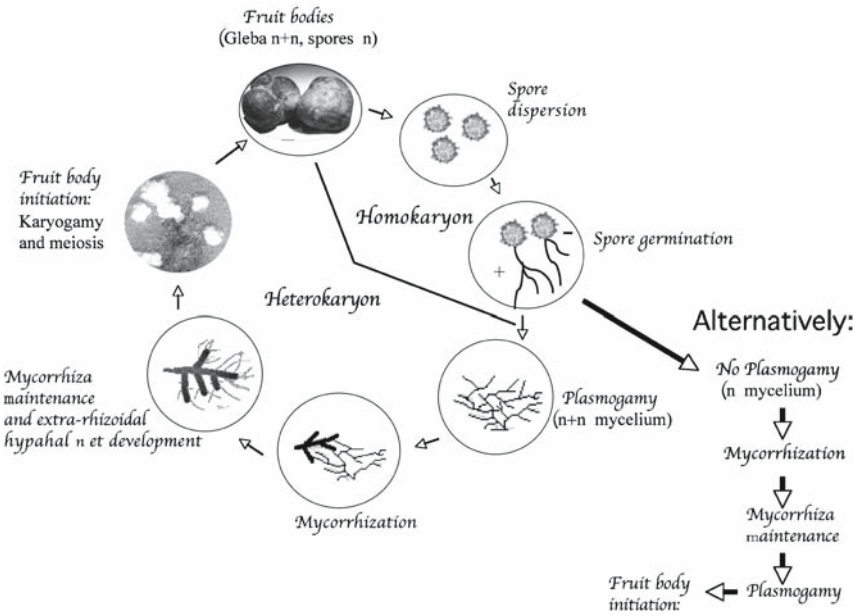

Fig. 4 A scheme of the life cycle of desert truffles consistent with available evidence

plants as a primary homokaryotic mycelium, or produces a secondary heterokaryotic mycelium after undergoing plasmogamy (i.e., before inoculation). It is not known precisely when plasmogamy occurs in the case of inoculation by a primary mycelium, or what the identity of the fertilizing complement donor is. However, fruit bodies are formed by heterokaryotic hyphae, as indicated by paired nuclei. Karyogamy and meiosis probably take place within the fruit body primordia (Roth-Bejerano et al. 2004).

3 Factors Determining the Type of Mycorrhiza Formed

As a general rule, all members of the same mycorrhitic fungal family will produce the same type of association in the wild, i.e. either ecto- or endomycorrhizal (leaving aside the ectendomycorrhizas). The desert truffles are exceptional in this respect: different species have been observed to engender different mycorrhizal types.

- Both *Terfezia leptoderma* (Chevalier et al. 1984; Dexheimer et al. 1985) and *Terfezia arenaria* (*leonis*) (Roth-Bejerano et al. 1990; and unpublished data) produce a Hartig net without mantle – essentially an underdeveloped ectomycorrhiza (Fig. 5) – in roots of different *Cistaceae* species.
- Endomycorrhizas lacking Hartig net and mantle but displaying undifferentiated intracellular hyphae have been observed in roots of *Helianthemum* species mycorrhized by *Terfezia boudieri* (Awameh 1981), *Terfezia boudieri, T. claveryi, Tirmania nivea* and *Tir. pinoyi* (Alsheikh 1984), and *Terfezia claveryi* (Dexheimer et al. 1985); as well as in roots of *Citrullus vulgaris* mycorrhized by *Kalaharituber pfeilii* (Fig. 6; Kagan-Zur et al. 1999).

Thus, the desert truffle family could be regarded as transitional between true ectomycorrhizal and true endomycorrhizal. However, the boundaries between ecto- and endomycorrhizal types are somewhat fluid, and the character of the mycorrhiza formed is often determined by external conditions:

- Haug et al. (1992), working on mycorrhizas of *Pisolithus tinctorius* in *Picea abies* roots, reported that high concentrations of either nitrate or ammonia resulted in a diminished Hartig net and in hyphal penetration into cortical cells. Brunner and Scheidegger (1994), studying colonization of the same plant species by *Hebeloma crustuliniforme*, reported that NH_4 media concentrations in excess of 30 mM caused a similar shift in mycorrhizal morphology, namely from the known wild-type ectomycorrhiza to an atypical endomycorrhiza.
- Gea et al. (1994) reported the ability of a high auxin-producing mutant of *Hebeloma cylindrosporum* to penetrate the cell wall of *Pinus pinaster* and form a kind of ectendomycorrhiza.

It may be worth noting that, so far at least, the shifts reported under altered environmental circumstances involved wild-type ectomycorrhizal fungi that turned ectendo- or endomycorrhizal – not the other way round – except for desert truffles.

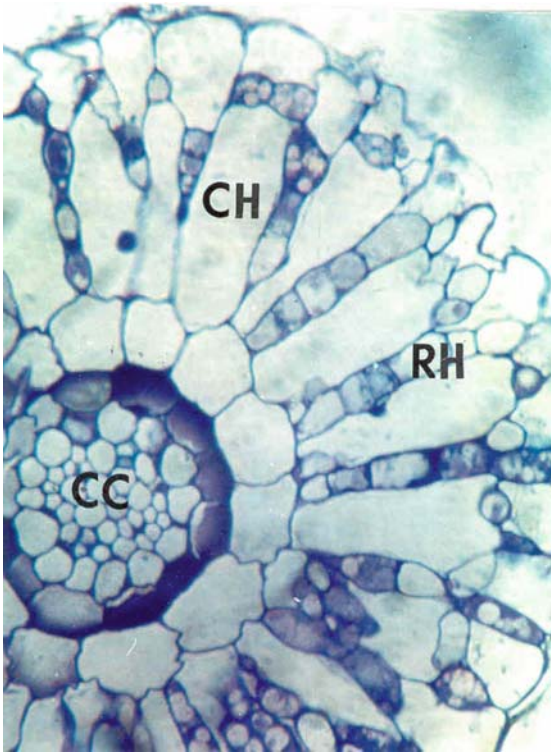

Fig. 5 Ectomycorrhiza of the desert truffle type. A transverse section of *Helianthemum guttatum* root mycorrhized by *Terfezia arenaria* under high phosphate concentration. Note lack of a mantle. From Fortas and Chevalier (1992), with kind permission of both Dr. Chevalier and the NRC Research Press

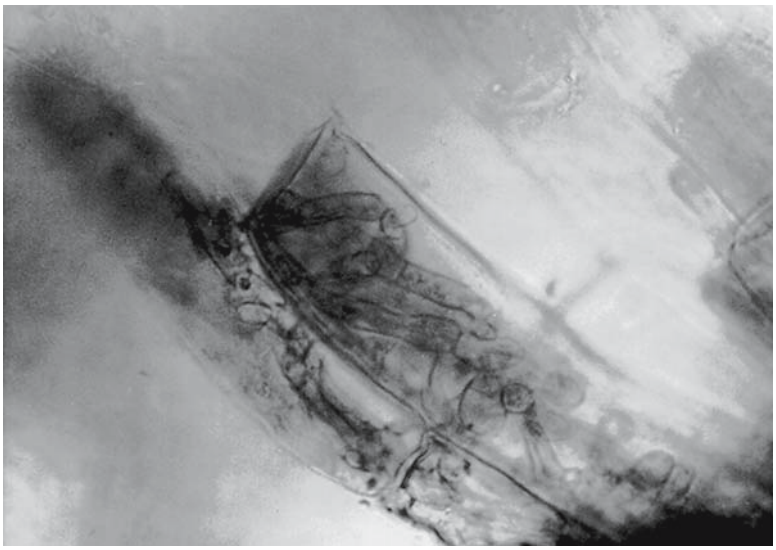

Fig. 6 Endomycorrhiza in *Citrullus vulgaris* root cells mycorrhized by *Kalaharituber pfeilii*. Magnification × 1000. Note septated undifferentiated hyphae within cells

Among the Pezizaceae, mycorrhization may be influenced by external factors:

- Fortas and Chevalier (1992) studied *Helianthemum guttatum* mycorrhized by *Terfezia arenaria, T. claveryi* or *Tirmania pinoyi* and found a shift from ectomycorrhizas at high phosphate (P) concentration (about 1.1 mM) to endomycorrhizas at low concentration (about 0.11 mM).
- Gutierrez et al. (2003), working with *Helianthemum almeriense* colonized by *Terfezia claveryi* and *Picoa lefebvrei*, encountered only endomycorrhizas under field conditions, only ectomycorrhizas in vitro (with a mantle, the only report of a mantle in a *Terfezia* mycorrhiza), and mostly ectomycorrhizas lacking a mantle in pots under greenhouse conditions. The environmental effectors leading to these differences were not studied.

In each of these systems, pairing of different fungal species with the same plant species led to similar mycorrhizal types, suggesting that genetic background plays no part. However, it has recently been demonstrated that both partners share in determining the type of mycorrhiza type formed (Zaretsky et al. 2006a).

Using an in vitro system in which several transformed root clones of *Cistus incanus* (Wenkart et al. 2001) were paired with different isolates of *Terfezia boudieri*, it was shown that the mycorrhiza obtained – endo- or ectomycorrhizal – depends on the following:

1. The identity of the fungal isolate, possibly modulated by auxin levels released into the medium (Table 1)
2. The identity of the root clone, possibly modulated by clonal sensitivity to auxin (Fig. 7)
3. The interaction between the concentration of phosphate (P) and that of available auxin in the growth medium (Fig. 8)

The importance of phosphate concentration for mycorrhizal establishment has long been recognized and is well documented (Harley and Smith 1983). Rantnayake

Table 1 Endogenous content of IAA in *C. incanus* clones and *T. boudieri* isolates as well as fungal IAA excretion. Values are the mean of at least three replicates ± standard error. Isolate 42a is capable of endomycorrhiza formation with clone M2 under low phosphate/low auxin concentrations, Isolate 27 is not (see Fig. 8). From Zaretsky et al. (2006a), with kind permission of Springer Science and Business Media

	Clone / Isolate	Free IAA (µg/g fw)	Conjugated IAA (IAA-Asp) (µg/g fw)
	Clone M2	0.121 ± 0.011	0.072 ± 0.045
	Clone W51	0.101 ± 0.009	0.076 ± 0.039
Endogenous IAA	Isolate 42a	0.064 ± 0.020	0.391 ± 0.140
	Isolate 27	0.256 ± 0.020	$0.035 \pm 1\ 10^{-4}$
IAA excretion	Isolate 42a	6.700 ± 1.140	14.600 ± 6.279
	Isolate 27	38.460 ± 7.950	538.900 ± 249.209

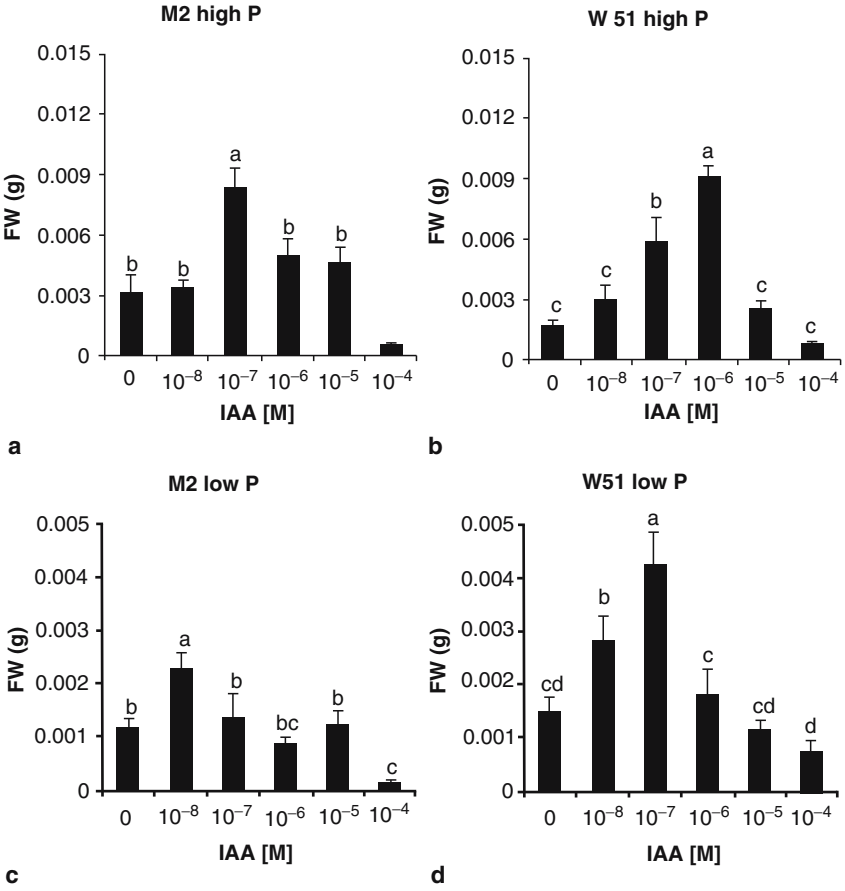

Fig. 7 Effect of exogenously applied IAA on growth of two transformed *C. incanus* hairy root clones (see Fig. 8). Roots were grown for 12 days on medium with a low (0.0035 mM) or high (0.70 mM) KH_2PO_4 concentration (*a,b* high P; *c,d* low P). IAA was added to the medium after autoclaving. Values are the mean of three plates containing three root tips each ± standard error per treatment. Different *letters* above columns indicate a significant difference ($P < 0.05$). Only the more sensitive clone M2 is capable of forming endomycorrhizas (with compatible *T. boudieri* isolates) under low phosphate/low auxin concentrations. From Zaretsky et al. (2006a), with kind permission of Springer Science and Business Media

et al. (1978) and Graham et al. (1981) provided at least a partial explanation by demonstrating that low phosphorus increases the permeability of the plasma membrane, leading to net loss of root metabolites; the amounts released are supposed to be sufficient to sustain germination and growth of the mycorrhizal fungus. However, researchers have only recently begun to examine how other effectors modify the action of P (see below).

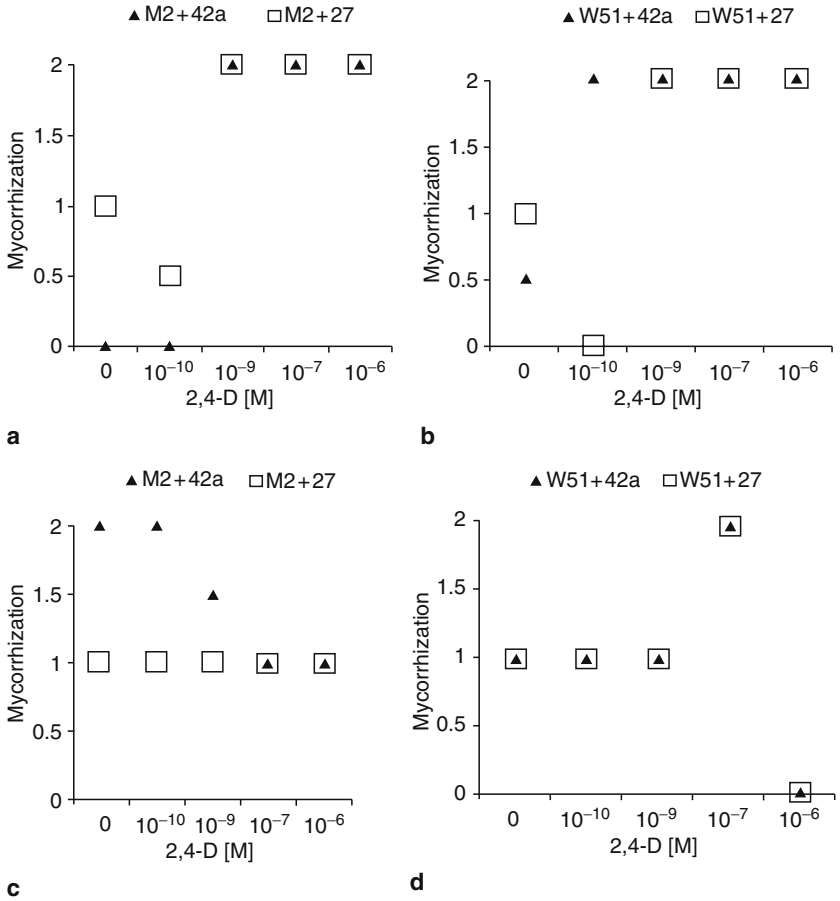

Fig. 8 Effect of exogenously applied 2,4-D on mycorrhiza formation. All plant–fungus couples were grown on media M modified with respect to KH_2PO_4 concentration (*a,b* high P; *c,d* low P). Roots were stained for assessment of endomycorrhiza. The type of mycorrhiza obtained was ranked by assigning arbitrary numbers as follows: no mycorrhiza (0), poor Hartig net (0.5), ectomycorrhiza with Hartig net (1), partly ecto- and partly endomycorrhiza (1.5), endomycorrhiza (2). From Zaretsky et al. (2006a), with kind permission of Springer Science and Business Media

Auxins, too, have long been known to play a role in ectomycorrhizal formation. Auxin has been shown to stimulate not only mycorrhizal establishment by *Hebeloma cylindrosporum* (Gay et al. 1994; Gea et al. 1994) but also mycorrhizal morphology, inducing formation of a multiseriate Hartig net occupying almost the entire root cortex. Auxin has been reported to alter plant cell wall extensibility through mechanisms involving decreased wall pH (Allen 1992), which could facilitate hyphal penetration of the plant cell wall. Changes of plant cell wall composition in response to auxin were also reported for the auxin-overproducing mutant of *Hebeloma* mentioned earlier (Gea et al. 1994).

It has been amply demonstrated that auxin can alter root morphology to create root structures recalling ectomycorrhizas (Barker and Tagu 2000), and several mycorrhizal fungi have been shown to secrete auxins (Barosso et al. 1978; Gay et al. 1994; Zaretsky et al. 2006a). Evidence exists that exogenous auxin up-regulates certain plant genes (Nehls et al.1998a; Charvet-Candela et al. 2002; Reddy et al. 2003). Martin and Tagu (1999) have suggested that auxin secreted by mycorrhizal fungi activates susceptible genes and that these in turn induce lateral root formation (Baser et al. 1987). In this context, it is interesting to note that, as has recently been shown, individual isolates of *T. boudieri* differ substantially in the amount of auxin they excrete; presumably they also elicit different responses from the plant root, responses that determine whether the symbiotic tissue will be ecto- or endomycorrhizal (Table 1; Zaretsky et al. 2006a).

A tie-in between auxin effects and phosphorus deficiency was reported by Lopez-Bucio et al. (2002). They found that P-deficient *Arabidopsis* plants were characterized by enhanced sensitivity to auxin, which was expressed in the development of short branched roots of the sort usually associated with mycorrhizas. This result is in accord with the finding of Zaretsky et al. (2006a) that elevated sensitivity to auxin enabled a low auxin excreting *T. boudieri* isolate to produce endomycorrhizas under conditions of phosphorus deprivation (0.0035 mM P). Earlier, in seeming contradiction to these results, Gea et al. (1994) had observed hyphal penetration into root cortical cells under high concentrations of both auxin and phosphate (2.27 mM P), which agrees with the more recent results reported by Zaretsky et al. (2006a) regarding the emergence of endomycorrhizas under high auxin coupled with high P (0.7 mM; Fig. 8). These seemingly contradicting results may point to a possibility that a certain auxin/phosphate ratio range is necessary for endomycorrhizas to be established – at least in fungal species capable of producing both ecto- and endomycorrhizal symbioses.

Other effectors can be involved in auxin-mediated mycorrhization. Herrmann et al. (2004) found that activated charcoal accelerated the progress of mycorrhization between oak and *Piloderma croceum* as did auxin. A study by Kagan-Zur et al. (1994) showed that activated charcoal removes iron from the growth solution, while iron deficiency stimulates mycorrhizal establishment. Interestingly, the effect of iron stress on root morphology is similar to that of auxin (Landsberg 1996). Either factor may affect mycorrhization independently, causing changes in root morphology that facilitate mycorrhization, though the possibility cannot be ruled out that, as in the case of auxin and phosphorus, there is an interaction between iron concentrations and auxin synthesis (Lopez-Bucio et al. 2002).

Phosphorus and nitrogen concentrations in the growth medium have been reported to affect IAA synthesis rates in the white rot fungus *Lentinus sajor-caju* (Yurekli et al. 2003). This finding linking nitrogen to auxin levels is of particular interest in light of the above mentioned reports by Haug et al. (1992) and Brunner and Scheidegger (1994) about high N concentrations causing a shift towards endomycorrhizas. It seems that here as well auxin plays a role in determining the type of mycorrhiza established. However, the effects of nitrogen on the course of mycorrhizal establishment (e.g., Hampp et al. 1999) – not to mention the effects of

other plant hormones and growth affecting substances known to affect other mycorrhizal relations (Gogala 1991; Beyrle 1995; Martin and Tagu 1999) – still await elucidation in the case of the desert truffles.

This much at least is clear: the host plant's sensitivity to auxin, auxin excretion by the fungus colonizer and nutrient availability, all exert some measure of control on what gene expression pathways will be activated and therefore on whether ecto- or endomycorrhizas will be established.

4 Genes Expressed in Fungus and Plant Prior to and under Mycorrhizal Conditions

For over a decade, researchers have been studying the changes in gene expression that take place as the free-living plant or fungus passes into the mycorrhizal state in endomycorrhizas (particularly VA mycorrhizas; see review by Balestrini and Lanfranco 2006) and in ectomycorrhizas. Gene expression studies may be designed to target specific genes or gene products (Kim et al. 1999; Nehls et al. 1998b, 1999; Wright et al. 2000). However, this assumes prior knowledge concerning the gene product or biochemical pathways under investigation, as well as the relevance of the studied system to the mycorrhizal process. Alternatively, overall approaches that require no previous knowledge or assumptions may be employed. Such explorations have been undertaken at two expression levels, that of mRNA (in the form of cDNA) (Voiblet et al. 2001; Podila et al. 2002; Sundaram et al. 2004) and that of protein (Hilbert et al. 1991; Burgess et al. 1995; Tarkka et al. 1998). Whatever the method of study, however, very little work has been done on gene expression changes during the establishement of mycorrhizas by truffles (Pierlioni et al. 2001; Polidori et al. 2002; Menotta et al. 2004; Miozzi et al. 2005), and only a single study directed at desert truffles (Zaretsky et al. 2006b) has been reported which will be discussed below.

Previously, tracking of gene expression changes corresponding to ecto- and endomycorrhizal establishment could not be undertaken due to the lack of suitable study systems. A possible way out of this dilemma was proposed in the recent experiments with the desert truffle *Terfezia boudieri* and *Cistus incanus* transformed roots referred to above (Zaretsky et al. 2006a), in which different clones of the same plant species were exposed to different isolates of the same fungal species, leading to the emergence of endomycorrhizas in some circumstances and combinations and ectomycorrhizas in others. We note parenthetically that some of these isolates were later discovered to be distinct cryptic species within the *T. boudieri* complex (Ferdman 2006), but this discovery was subsequently found to be irrelevant to the ability of the isolates to form endomycorrhizas with the endomycorrhiza-capable clone.

The new study system was subsequently used to identify genes expressed in *Terfezia boudieri* during pre-infection and in the mycorrhizal state in the course of establishment of ecto- and endomycorrhizas (Zaretsky et al. 2006b). The study was

conducted under low P (0.0035 mM) conditions, which have been shown to favor manifestation of root clone–fungal isolate effects. The use of two different *T. boudieri* isolates in conjunction with two different *C. incanus* hairy root clones made it possible to detect differences in gene expression in the same fungal isolate in response to different plant clones, as well as differences in gene expression between individual fungal isolates in response to the same plant clone (Fig. 9). Shifts of gene expression pattern were, of course, the rule in all the transitions studied. For example, genes expressed under pre-infection conditions differed from those expressed under mycorrhiza-enabling conditions.

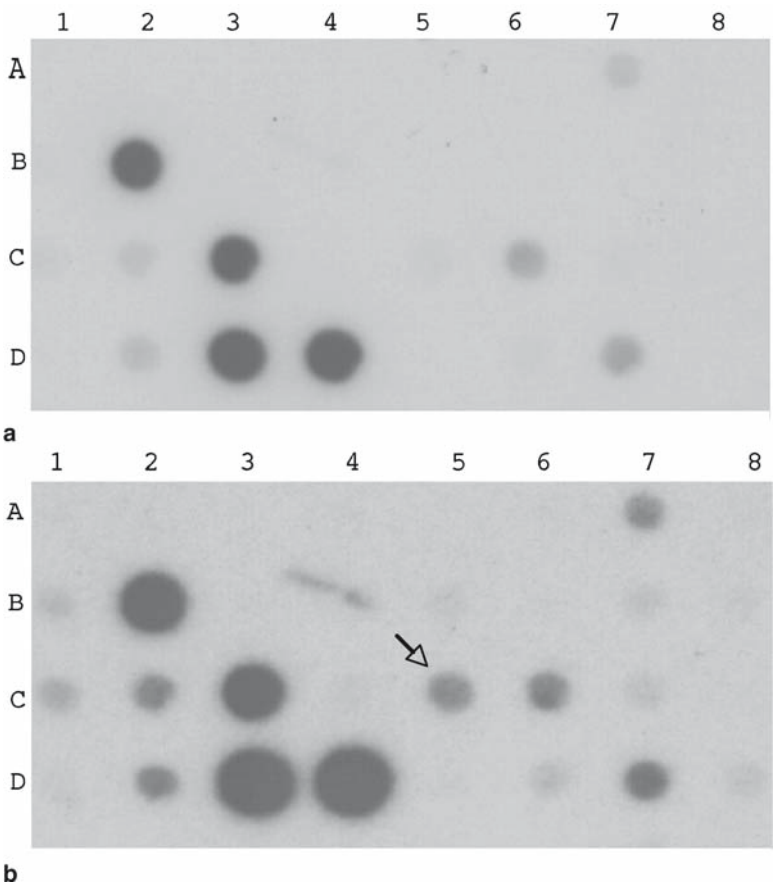

Fig. 9 Two representative membranes subjected to reverse Northern analysis (dot blot, macroarray). Each membrane contains 32 *T. boudieri* cDNA fragments isolated from cDNA-AFLP gels and spotted on rows *A, B, C* and *D*, columns *1–8*. Membrane **a**: cDNA was synthesized from RNA extracted from fungal isolate 42a separated by cellophane from plant clone M2 (pre-infection stage) and used as a probe for hybridization. Membrane **b**: cDNA was synthesized from RNA extracted from fungal isolate 42a separated by cellophane from plant clone W51 (pre-infection stage) and used as a probe for hybridization

In this study, 260 mycorrhiza-associated cDNA-expressed sequence tags (EST) were cloned from *T. boudieri* by the cDNA-AFLP method (Vos et al. 1995), out of the more than 300 tags detected. Most of the non-cloned fragments were expressed solely in cDNA from free-living cultures and were downregulated at the pre-mycorrhizal or mycorrhizal stages. In comparison, only 75 ESTs from *C. incanus* could be identified as mycorrhiza associated (out of 150 differentially expressed fragments). After discarding fragments uniformly expressed both during pre-infection and at the mycorrhiza stage, as well as ESTs that failed to be expressed at all, and following cluster analysis, 63 independent reporter fungal ESTs were left versus only 10 plant ones (about half of the number expected in view of the fungal results). Of these 10 not even 1 was expressed solely under pre-infection conditions, which agrees with reports (Hilbert et al. 1991; Burges et al. 1995) of large-scale downregulation of root protein synthesis during ectomycorrhiza establishment.

Three groups of fungal genes were found to be involved in mycorrhizal establishment: (1) transiently expressed genes (expressed only under pre-infection conditions); (2) genes expressed solely under mycorrhiza-enabling conditions; and (3) genes expressed in both situations. The latter exhibited altered expression patterns with respect to preinfection and mycorrizal conditions (Zaretsky et al. 2006b).

Expressed genes (identified through homology to genes in the GenBank) belonged to the following groups:

1. Genes involved in metabolism (carbohydrate and amino acid metabolism, sucrose metabolism, etc.); similar genes have been shown to be expressed in *Eucalyptus-Pisolithus* ectomycorrhizas (Voiblet et al. 2001; Duplessis et al. 2005).
2. Genes involved in vesicular trafficking (vacuolar proteins) and membrane transport, such as ATP-binding cassette, ABC and cation transporters (Menotta et al. 2004).
3. Genes involved in signal transduction pathways (genes with homology to Ras and GTP-binding proteins), as shown by Krüger et al. (2004) for plant genes expressed at pre-ectomycorrhizal stages between *Quercus robur* and *Plodder croceum*. The cross talk between the symbionts is apparently mediated by these genes (Podila et al. 2002).
4. Genes involved in protein synthesis and processing (rRNA genes) and gene-expression pathways, which may regulate the metamorphic changes that take place during mycorrhizal development (Podila et al. 2002).

However, the largest group by far consisted of fragments for which no match could be found in the GenBank. Voiblet et al. (2001) and Duplessis et al. (2005) also reported the isolation of expressed sequence tags lacking GenBank matches.

When paired with the same root clone, and regardless of whether that clone was endomycorrhizas-supporting or supported only ectomycorrhizal associations, the two *T. boudieri* isolates displayed quite different gene complements. Le Quéré et al. (2004) also demonstrated that, in two closely related strains of *Paxillus involutus*, different isolates expressed different gene complements when paired with the same host. Thus, the fact that a given gene is present (or absent) only in response to

the single root clone-fungus combination capable of forming endomycorrhiza is not convencing enough. Therefore in order for a gene to be characterized as involved in the type of mycorrhiza formed, it has to occur consistently in all root–fungus combinations expected to produce ectomycorrhizas while being absent in the one combination expected to result in an endomycorrhiza or vice versa (Table 2).

Only 11 fungal ESTs and a single plant EST (12 altogether) met these requirements. Five were expressed solely under pre-infection conditions, five solely under mycorrhiza-enabling conditions (including the plant fragment), and two under both sets of conditions. However, the latter were endomycorrhiza-specific under mycorrhiza-enabling conditions.

From these results, it is apparent that the genes involved in the production of ecto- or endomycorrhizas are expressed at different stages: genes that are ectomycorrhiza-specific under pre-infection conditions may exhibit a different mode of expression under conditions permitting mycorrhiza formation.

For none of the 12 ectomycorrhiza-specific genes could a matching sequence be found in the GenBank. This could have several causes:

1. The sequences represent 5’ or 3’ untranslated regions (5’ or 3’ UTR).
2. The cloned fragments are too short to yield an acceptable *e* value.
3. Several ectomycorrhizal studies have identified fungal (Voiblet et al. 2001; Podila et al. 2002) or plant (Voiblet et al. 2001; Krüger et al. 2004) genes showing no similarity to any sequences in the NCBI database. Podila et al. (2002) have suggested that the expression of these genes, or the genes themselves, may be unique to the particular ectomycorrhizal fungus studied, or that they may

Table 2 Types of gene expression patterns encountered

Gene expression combination associated with	Isolate/clone pairs				Number of ESTs	
	42a / M2	27 / M2	42a/W51	27/W51	Pre-infection	Mycorrhiza
Isolate 42a	+	–	+	–	–	1[a]
Isolate 27	–	+	–	+	18	2
Clone M2	+	+	–	–	2	1
Clone W51	–	–	+	+	1[a]	–
All isolates and clone pai rs	+	+	+	+	9	1
Ectomycorrhizas	–	+	+	+	5	4+2[a]
Endomycorrhizas	+	–	–	–	2[a]	–
A single isolate/ clone pair	–	+	–	–	2	1

[a] ESTs expressed at both the pre-infection and the mycorrhizal stage. 17 ESTs were expressed at both stages. All had different expression patterns at each stage (34 isolate/clone pair expression patterns altogether); for example, the two ecto- and two endo- expressed clones marked ([a]) are the same clones, differently expressed at the two stages. To avoid confusion, only 6 of the 34 patterns were chosen to be marked in this table

represent very rare transcripts that have not been identified and/or characterized previously. However, it is also possible that these sequences represent unidentified fungal mycorrhiza-specific genes.

Eliminating the first two possibilities necessitated extending the existing fragments to obtain a full length of each gene. Extension of four of the mycorrhiza-specific genes was undertaken (1 expressed solely under pre-infection, 1 expressed under both conditions – ecto- under pre-infection, endo- in mycorrhizas – and 2 expressed solely under the mycorrhiza enabling conditions). After extension, GenBank matches were found for three of the four. Two of the originally cloned sequences proved to be part of the UTR, while the third lay within the coding sequence but had originally been too short to give an acceptable *e* value. The fourth, expressed under pre-infection conditions, was a short peptide with no match in the databases. In the single isolate found to be incapable of forming endomycorrhizas under our study conditions, this peptide was two amino acids shorter than in the isolate capable of such associations (Table 3, Fig. 10). It is becoming increasingly evident that peptides play an important role in many biological processes (Negishi et al. 1995; Raaijmakers et al. 2006; Selth et al. 2006; Mygind et al. 2005). So far, however, there has been no report of a peptide with a definite role in mycorrhiza establishment or maintenance.

At this point we cannot be sure that other isolates – endomycorrhiza forming or not – will express this specific peptide-gene under the same conditions, nor do we know whether and how this gene product(s) interact(s) with P and auxin levels to affect mycorrhizal type determination. However, it may be part of a signal transduction pathway yet to be elucidated.

Table 3 Sequences of short peptides isolated from cDNA and genomic DNA of isolates 42a and 27. Isolate 42a codes for a peptide 2 amino acids longer (shaded in dark grey) than that of isolate 27

Source	Peptide sequence
Isolate 27 cDNA (GenBank accn.. no.DQ912827) Genomic DNA (GenBank accn.. no. DQ912826)	M N T I V T R Q G R G V M V G G G G A G M N
Isolate 42a cDNA (GenBank accn.. no. DQ903321) Genomic DNA (GenBank accn.. no. DQ912825	M N T I V T R Q G R G V M V G G G G A G M N Y R)

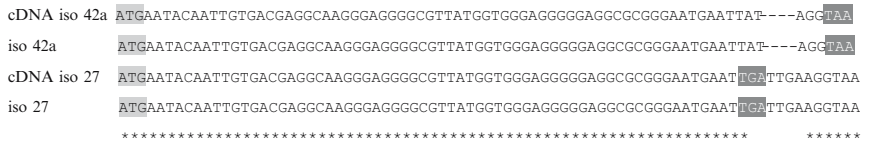

Fig. 10 Sequence alignment of the peptide coding regions in isolates 42a and 27. START codon (ATG) is shaded in *light grey*, while the *dark grey* shading designates a STOP codon in the sequences

5 Conclusions

As we saw, the hypogeous members of the Pezizaceae family have been largely neglected, so much so that much about their life cycle remains obscure.

From a scientific point of view, the desert truffle group of mycorrhizal fungi is well worth investigating. Its ectomycorrhizal associations in the wild lack a mantle, and its endomycorrhizal associations are comprised of undifferentiated intracellular hyphae, features that place it somewhere between true ecto- and true endomycorrhiza forming families. This unique situation should harbor important and intriguing research assets.

Long an arena of random (and surprising) discoveries, the external factors involved in setting the future course of mycorrhizal development are now beginning to receive goal-directed research attention. A good deal more will have to be done before the underlying pathways and control mechanisms are properly understood. To mention just a few outstanding problems. Can all nutrient effects be summed up in terms of an auxin/nutrient ratio? How are the effects of nutrients such as iron, phosphate or nitrogen interrelated in the context of mycorrhizal determination? What is the role of the other plant hormones that have been shown to be produced by ectomycorrhizal fungi (Gogala 1991)? A study by Hirsch et al. (1997) indicates that cytokinins may be involved in mycorrhizal establishment by AM fungi, but no proof exists of their involvement in the formation of ectomycorrhizas (Barker and Tagu 2000), let alone in controlling the type of mycorrhiza formed. Regarding the changes in root morphology induced by the different effectors, what is the threshold that triggers each mycorrhizal type?

Moreover, this group of hypogeous mycorrhizal members of the Pezizaceae provides a very good starting point for seeking out genes associated with ecto- and endomycorrhizal establishment, particularly the genes involved in controlling each process and those that are expressed at the junction point between the two types. In addition, analysis of the upstream transcription-controlling sequences that differentiate between fungal isolates capable and incapable of forming endomycorrhizas under low P and low auxin should afford important insights into mycorrhizal control motifs, thereby advancing general mycorrhizal research. But this is just the beginning, and at this stage it is difficult to predict the lines along which future research will progress.

One last remark: the life cycle of this group of truffles seems to differ from that of the other Ascomycetes. It would be useful to determine the exact timing of plasmogamy, karyogamy and meiosis among the desert truffles, a knowledge of which might help explain the consistent finding of paired nuclei in long-term mycelia derived from fruit body gleba.

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References

Ackerman LGJ, Van Wyk PJ, du Plessis LM (1975) Some aspects of the composition of the Kalahari truffle or N'abba. S Afr Food Rev 2:145–146

Ahmed-Ashour A, Mohamed MA Hami MA (1981) Lybian truffles: chemical composition and toxicity. Mushroom science XI. Part II. In: Proceedings of the 11th International Congress on the Cultivation of Edible Fungi. Sydney, Australia, pp 833–842

Al-Delaimy KS (1977) Protein and amino acid composition of truffles. Can Inst Food Sci Technol J 10:221–222

Al-Shabibi MMA, Toma SJ, Hadad BA (1982) Studies on Iraqi truffles. I. Proximate analysis and characterization of lipids. Can Inst Food Sci Technol J 15:200–202

Alexopoulos CJ, Mims CW, Blackwell M (1996). Introductory mycology IV. Wiley, New York

Allen MJ (1992). Mycorrhizal functioning: an integrative plant–fungal process. Routledge, Chapman & Hall, New York

Alsheikh, A (1984). Mycorrhizae of annual *Helianthemum* species formed with desert truffles. Proceedings 6th North American Conference on Mycorrhiza, Bend, Oregon. Oregon State University, Corvallis, Or., no. 445

Aviram S, Roth-Bejerano N, Kagan-Zur V (2004) Two *ITS* forms co-inhabiting a single genet of an isolate of *Terfezia boudieri* (Ascomycotina), a desert truffle. Anton Leeuw Int J 85:169–174

Awameh MS (1981) The response of *Helianthemum salicifolium* and *H. ledifolium* to the infection by the desert truffle *Terfezia boudieri*. Mushroom science. XI. Part II. In: Proceedings of the 11th International Congress on the Cultivation of Edible Fungi, Sydney, Australia, pp 843–583

Balestrini R, Lanfranco L (2006) Fungal and plant gene expression in arbuscular mycorrhizal symbiosis. Mycorrhiza 16:509–524

Barker SJ, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbiosis. J Plant Growth Regul 19:144–154

Barosso J, Chaves Neves H, Pais MS (1978) Production of of indole-3-ethanol and indole-3-acetic acid by the mycorrhizal fungus of *Ophris lutea* (Orchidaceae). New Phytol 103:745–749

Baser CM, Garrett HE, Mitchell RJ, Cox GS (1987) Indole butyric acid and ectomycorrhizal inoculation increase lateral root initiation and development of container grown black oak seedlings. Can J For Res 17:36–39

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D (1998) Trifling variation in truffles. Nature 394:734

Bertault G, Rousset F, Fernandez D, Berthomieu A, Hochberg ME, Callot G, and Raymond M (2001) Population genetics and dynamics of the black truffle in a man-made truffle field. Heredity 86:451–458

Beyrle H (1995) The role of phytohormones in the function and biology of mycorrhizas. In: Varma AK and Hock B (eds) Mycorrhiza: structure, molecular biology and function. Springer, Heidelberg, pp 365–390

Bokhari A, Parvez S (1993) Chemical composition of desert truffles *Terfezia claveryi*. J. Food Comp Anal 6:285–293

Bokhari YHA, Suleiman AAA, Basalah MO (1989) Fatty acid composition of the desert truffle "Al-Kamah" of Saudi Arabia J. Food Prot 52:668–669

Bonfante-Fasolo P, Brunel A (1972) Caryological features in a mycorrhizal fungus: *Tuber melanosporum* Vitt. Allionia 18:5–11

Brunner I, Scheidegger C (1994) Effects of high nitrogen concentrations on ectomycorrhizal structure and growth of seedlings of *Picea abies* (L.) Karst. New Phytol 129:83–95

Burgess T, Laurent P, Dell B, Malajczuk N, Martin F (1995) Effect of fungal isolate infectivity on the biosynthesis of symbiosis-related polypeptides in differentiating eucalypt ectomycorrhizas. Planta 195:408–417

Charvet-Candela V, Hitchin S, Ernst D, Sandermann H Jr, Marmeisse R, Gay G (2002) Characterization of an *Aux/IAA* cDNA upregulated in *Pinus pinaster* roots in response to colonization by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. New Phytol 154:769–777

Chevalier G, Riousset L, Dexheimer J, Dupre C (1984) Synthese mycorhizienne entre *Terfezia leptoderma* Tul. et diverses Cistaceaes. Agronomie 4:210–211

Dexheimer J, Gerard J, Leduc JP, Chevalier G (1985) Etude ultrastructurale compar e des associations symbiotiques mycorhiziennes *Helianthemum salicifolium – Terfezia claveryi* et *Helianthemum salicifolium – Terfezia leptoderma*. Can J Bot 63:582–591

Diez J, Manjon JL, Martin F (2002) Molecular phylogeny of the mycorrhizal desert truffles (*Terfezia* and *Tirmania*), host specificity and edaphic tolerance. Mycologia 94:247–259

Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299:111–117

Dover G (1986) Molecular drive in multigene families: how biological novelties arise, spread and are assimilated. Trends Genet 2:159–165

Duplessis S, Courty P-E, Tagu D, Martin F (2005) Transcript patterns associated with ectomycorrhiza development in *Eucalyptus globulus* and *Pisolithus microcarpus*. New Phytol 165: 599–611

Ferdman Y (2006) Phylogenetic relationships and cryptic species in desert truffles. PhD thesis, Dept Life Sci., Ben-Gurion University. (In Hebrew, English abstract)

Ferdman Y, Aviram S, Roth-Bejerano N, Trappe JM, Kagan-Zur V (2005) Phylogenetic studies of *Terfezia pfeilii* and *Choiromyces echinulatus* (Pezizales) support new genera for southern African truffles: *Kalaharituber* and *Eremiomyces*. Mycol Res 109:236–244

Fortas Z, Chevalier G (1992) Effet des conditions de culture sur la mycorhization de l'*Helianthemum guttatum* par trois especes de terfez des genres *Terfezia* et *Tirmania* d'Algerie. Can J Bot 70:2453–2460

Frizzi, G, Lalli G, Miranda M, Pacioni G (2001) Intraspecific isozyme variability in Italian populations of the white truffle *Tuber magnatum*. Mycol Res 105:365–369

Ganley ARD, Scot B (1998) Extraordinary ribosomal spacer length heterogeneity in a Neotyphodium endophyte hybrid: implications for concerted evolution. Genetics 150:1625–1637

Gay G, Normand L, Marmeissa R, Sotta B, Debaud JC (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. New Phytol 128:645–657

Gea L, Normand L, Vian B, Gay G (1994) Structural aspects of ectomycorrhiza of *Pinus pinaster* (Ait.) Sol. formed by an IAA-overproducer mutant of *Hebeloma cylindrosporum* Romagnesi. New Phytol 128:659–670

Glass NL, Jacobson DJ, Shiu PKT (2000) The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. Annu Rev Genet 34:165–186

Gogala N (1991) Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi. Experientia 47:331–340

Graham JH, Leonard RT, Menge JA (1981) Membrane–mediated decrease in root exudation responsible for phosphorus inhibition of vesicular arbuscular formation. Plant Physiol 68:548–552

Gutierrez A, Morte A, Honrubia M (2003) Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* with *Terfezia clavery* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorrhiza 13:299–307

Hampp R, Wiese J, Mikolauewska S. Nels U (1999) Biochemical and molecular aspect of C/N interaction in ectomycorrhizal plants: an update. Plant Soil 215:103–113

Hansen K, Læssøe T, Pfister DH (2001) Phylogenetics of the Pezizaceae, with emphasis on Peziza. Mycologia 93: 958–990

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic, London

Haug S, Ritter T, Kottke I, Oberwinkler F (1992) Steigerung der Leistungsfahigkeit von Forstpflanzen (*Fagus silvatica* L. und *Quercus robur* L.) durch kontrollierte Mykorrhizierung. Allg Forst Jagdztg 163:72–79

Herrmann S, Oelmuller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis J Plant Physiol 161:509–517

Hilbert JL, Costa G, Martin F (1991) Ectomycorrhizin synthesis and polypeptid changes during the early stage of eucalypt mycorrhiza development. Plant Physiol 97:977–984

Hirsch AM, Fang Y, Asad S, Kapulnik Y (1997) The role of phytohormones in plant-microbe symbioses Plant Soil 194:171–184

Honrubia M, Gutierrez A, Morte A (2002) Desert truffle plantation from south-east Spain. Edible mycorrhizal mushrooms and their cultivation. In: Hall IR, Wang Y, Danell E, Zambonelli A (eds) Proceedings of the 2nd International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3–6 July 2001. New Zealand Institute for Crop & Food Research Limited, Christchurch CD edition

Kagan-Zur V, Raveh E, Lischinsky S, Roth-Bejerano N (1994) *Helianthemum - Terfezia* association is enhanced by low iron in the growth medium. New Phytol 127:567–570

Kagan-Zur V, Kuang J, Tabak S, Taylor FW, Roth-Bejerano N (1999) Potential verification of a host plant for the desert truffle *Terfezia pfeilii* by molecular methods. Mycol Res 103:1270–1274

Kim SJ, Bernreuther D, Thumm M, Podila GK (1999) LBAUT7, a novel symbiosis-regulated gene from an ectomycorrhizal fungus, *Laccaria bicolor*, is functionally related to vesicular transport and autophagocytosis. J Bacteriol 181:1963–1967

Krüger A, Peskan-Berghöfer T, Frettinger P, Herrmann S, Buscot F, Oelmüller R (2004) Identification of premycorrhiza-related plant genes in the association between *Quercus robur* and *Piloderma croceum*. New Phytol 163:149–157

Landsberg EC (1996) Hormonal regulation of iron-stress response in sunflower roots: A morphological and cytological investigation. Protoplasma 194:69–80

Le Quéré A, Schützendübel A, Rajashekar B, Canbäck B, Hedh J, Erland S, Johansson T, Tunlid A (2004) Divergence in gene expression related to variation in host specificity of an ectomycorrhizal fungus. Molecular Ecology 13:3809–3819

López-Bucio J, Hernández-Abreu E, Sánchez-Caldéron L, Nieto-Jacobo M, Simpson J, Herrera-Estrella L (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. Plant Physiol 129:244–256

Martin F, Tagu D (1999) Developmental biology of a plant-fungus symbiosis: ectomycorrhizal In: Varma A, Hock, B (eds) Mycorrhiza, 2nd edn. Springer, Heidelberg, pp 51–73

Mello A, Murat C, Vizzini A, Gavazza V, Bonfante P (2005) *Tuber magnatum*, a species of limited geographical distribution: its genetic diversity inside and outside a truffle ground. Environ Microbiol 7:55–65

Menotta M, Amicucci A, Sisti D, Gioacchini AM, Stocchi V (2004) Differential gene expression during pre-symbiotic interaction between *Tuber borchii* Vittad. and *Tilia americana* L. Curr Genet 46:158–165

Miozzi L, Balestrini R, Bolchi A, Novero M, Ottonello S, Bonfante P (2005) Phospholipase A(2) up-regulation during mycorrhiza formation in *Tuber borchii*. New Phytol 167:229–238

Murat C, Diez J, Luis P, Delaruelle C, Dupre C, Chevalier G, Bonfante P, Martin F (2003) Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Perigord truffle *Tuber melanosporum*. New Phytol 164:401–411

Murcia MA, Martínez-Tomé M, Vera AM, Morte A, Gutiérrez A, Honrubia M, Jiménez AM (2003) Effect of industrial processing on desert truffles *Terfezia claveryi* Chatin and *Picoa juniperi* Vittadini: proximate composition and fatty acids. J Sci Food Agric 83:535–541

Mygind PH, Fischer RL, Schnorr KM, Hansen MT, Sonksen CP, Ludvigsen S, Raventos D, Buskov S, Christensen B, De Maria L, Taboureau O, Yaver D, Elvig-Jorgensen SG, Sorensen MV, Christensen BE, Kjaerulff S, Frimodt-Moller N, Lehrer RI, Zasloff M, Kristensen HH (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. Nature 437:975–980

Negishi I, Motoyama N, Nakayama K, Senju S, Hatakeyama S, Zhang Q, Chan AC, Loh DY (1995) Essential role for Zap-70 in both positive and negative selection of thymocytes. Nature 376:435–438

Nehls U, Beguiristain T, Ditengou F, Lapyrie F, Martin F (1998a) The expression of a symbiosis regulated gene in Eucalypt roots is regulated by auxins and hypaphorine, the tryptophane betaine of the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. Planta 207:296–302

Nehls U, Wiese J, Guttenberger M, Hampp R (1998b) Carbon allocation in ectomycorrhizas: identification and expression analysis of an *Amanita muscaria* monosaccharide transporter. Mol Plant Microb Interact 11:167–176

Nehls U, Mikolajewski S, Ecke M, Hampp R (1999) Identifcation and expression-analysis of two fungal cDNAs regulated by ectomycorrhiza and fruit body formation. New Phytol 144:195–202

Norman JE, Egger KN (1999) Molecular phylogenetic analysis of Peziza and related genera. Mycologia 91:820–829

O'Donnell K, Cigelnic E, Weber NS, Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89:48–65

Paolocci F, Rubini A, Riccioni C, Arcioni S (2006) Reevaluation of the life cycle of *Tuber magnatum*. Appl Environ Microbiol 72:2390–2393

Percudani R, Trevisi A, Zambonelli A, Ottonello S (1999) Molecular phylogeny of truffles (Pezizales: Terfeziaceae, Tuberaceae) derived from nuclear rDNA sequence analysis. Mol Phylogenet Evol 13:169–180

Pérez-Gilabert, M, Morte A, Hornubia M, García-Carmona F (2001a) Monophenolase activity of latent *Terfezia claveryi* tyrosinase: characterization and histochemical localization. Physiol Plant 113:203–209

Pérez-Gilabert M, Morte A, Honrubia M, García-Carmona F (2001b) Partial purification, characterization and histochemical localization of fully latent desert truffle (*Terfezia claveryi* Chatin) polyphenol oxidase. J Agric Food Chem 49:1922–1927

Pérez-Gilabert M, Morte A, García-Carmona F (2004) Histochemical and biochemical evidences of the reversibility of tyrosinase activation by SDS. Plant Sci 166:365–370

Pérez-Gilabert M, Sánchez-Felipe I, Morte A, García-Carmona F (2005) Kinetic properties of lipoxygenase from desert truffle (*Terfezia claveryi* Chatin) ascocarps: effect of inhibitors and activators. J Agric Food Chem 53:6140–6145

Pierleoni R, Vallorani L, Sacconi C, Sisti D, Giomaro G, and Stocchi V (2001) Evaluation of the enzymes involved in primary nitrogen metabolismin of *Tilia platyphyllos-Tuber borchii* ectomycorrhizae. Plant Physiol Biochem 39:1111–1114

Podila G, Zheng J, Balasubramanian S, Sundaram S, Hiremath S, Brand JH, Hymes MJ (2002) Fungal gene expression in early symbiotic interactions between *Laccaria bicolor* and red pine. Plant Soil 244:117–128

Polidori E, Agostini D, Zeppa S, Potenza L, Palma F, Sisti D, Stocchi V (2002) Identification of differentially expressed cDNA clones in *Tilia platyphyllos–Tuber borchii* ectomycorrhizae using a differential screening approach. Mol Genet Genom 266: 858–864

Raaijmakers JM, de Bruijn I, de Kock MJD (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: Diversity, activity, biosynthesis, and regulation. Mol Plant Microb Interact 19:699–710

Rantnayake M, Leonard RT, Menge JA (1978) Root exudation in relation to supply of phosphorus and its possible relevance to mycorrhizal formation New Phytol 81:543–552

Reddy SM, Pandey AK, Melayah D, Marmeisse R, Gay G (2003) The auxin responsive gene *Pp-C61* is up-regulated in *Pinus pinaster* roots following inoculation with ectomycorrhizal fungi Plant Cell Environ 26:681–691

Roth-Bejerano N, Livne D Kagan-Zur V (1990) *Helianthemum-Terfezia* relations in different growth media. New Phytol 114:235–238.

Roth-Bejerano N, Li Y-F, Kagan-Zur V (2004) Homokaryotic and heterokaryotic hyphae in *Terfezia*. Anton Leeuw Int J 85:165–168

Roux C, Sejalon-Delmas N, Martins M, Parguey-Leduc A, Dargent R, Becard G (1999) Phylogenetic relationships between European and Chinese truffles based on parsimony and distance analysis of ITS sequences. FEMS Microbiol Lett 180:147–155

Sakri FA (1989) Chemical composition and mineral content of Iraqi truffles. Iraqi J Sci 30:421–424

Sawaya WN, Al-Shalhat A, Al-Sogar A, Al-Mohammed M (1985) Chemical composition and nutritive vlaue of truffles of Saudi-Arabia. J Food Sci 50:450–453

Selth LA, Dogra SC, Rasheed MS, Randles JW, Rezaian MA (2006) Identification and characterization of a host reversibly glycosylated peptide that interacts with the tomato leaf curl virus V1 protein. Plant Mol Biol 61:297–310

Sundaram S, Brand JH, Hymes MJ, Hiremath S, Podila GK (2004) Isolation and analysis of a symbiosis-regulated and Ras-interacting vesicular assembly protein gene from the ectomycorrhizal fungus *Laccaria bicolor*. New Phytol 161:529–538

Tarkka M, Niini SS, Raudaskoski M (1998) Developmentally regulated proteins during differentiation of root system and ectomycorrhiza in Scotspine (*Pinus sylvestris*) with *Suillus bovinus*. Physiol Plant 104:449–455

Tedersoo L, Hansen K, Perry BA, Kjoller R (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytol 170:581–596

Trappe JM (1979) The orders, families, and genera of hypogeous Ascomycotina (truffles and their relatives). Mycotaxon 9:297–340

Urban A, Neuner-Plattner I, Krisai-Greilhuber I, Haselwandter K (2004) Molecular studies on terricolous microfungi reveal novel anamorphs of two *Tuber* species. Mycol Res 108:749–758

Voiblet C, Duplessis S, Encelot N, Martin F (2001) Identification of symbiosis-regulated genes in *Eucalyptus globules–Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 25:181–191

Volk TJ, Leonard TJ (1990) Cytology of the life cycle of Marcella. Mycol Res 94:399–406

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acid*s* Res 23:4407–4414

Wenkart S, Roth-Bejerano N, Mills D, Kagan-Zur V (2001) Mycorrhizal associations between *Tuber melanosporum* mycelia and transformed roots of *Cistus incanus*. Plant Cell Rep 20:369–373

Wright DP, Scholes JD, Read DJ, Rolfe SA (2000) Changes in carbon allocation and expression of carbon transporter genes in Betula pendula Roth. colonized by the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. Plant Cell Env 23:39–49

Yurekli F, Geckil H, Topocuogli F (2003) The synthesis of indole-3-acetic acid by the industrially important white-rot *Lentinus dajor-caju* under different culture conditions Mycol Res 107:305–309

Zaretsky M, Kagan-Zur V, Mills D, Roth-Bejerano N (2006a) Analysis of mycorrhizal associations formed by *Cistus incanus* transformed root clones with *Terfezia boudieri* isolates. Plant Cell Rep 25:62–70

Zaretsky M, Sitrit Y, Mills D, Roth-Bejerano N, Kagan-Zur V (2006b) Differential expression of fungal genes at preinfection and mycorrhiza establishment between *Terfezia boudieri* isolates and *Cistus incanus* hairy root clones. New Phytol 171:837–846

Evaluation of the Possible Participation of Drought-induced Genes in the Enhanced Tolerance of Arbuscular Mycorrhizal Plants to Water Deficit

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1 Introduction

Drought stress is considered one of the most important abiotic factors that interferes with normal development and growth of plants, having a major adverse effect on plant survival and productivity (Kramer and Boyer 1997; Bray 2004). However, plants can respond to drought stress at morphological, anatomical and cellular levels with modifications that allow the plant to avoid the stress or to increase its tolerance (Bray 1997). The morphological and anatomical adaptations can be of vital importance for some plant species, but they are not a general response of all plant species. In contrast, the cellular responses to water deficit seem to be conserved in the plant kingdom. Plants can adapt to water deficit by the induction of specific genes such as genes encoding proteins involved in the biosynthesis of osmoregulatory compounds, genes encoding late embryogenesis abundant (LEA) proteins, genes encoding proteins with chaperone activity such as proteins 14-3-3 or binding proteins (BiPs), as well as modulating the expression of genes encoding aquaporins (AQP) (Zhu et al. 1997; Alvim et al. 2001; Zhu 2002; Wang et al. 2003; Bray 2004; Luu and Maurel 2005).

In addition to the intrinsic protective systems of plants against water deficit, most terrestrial plants can also establish a symbiotic association with arbuscular mycorrhizal (AM) fungi. The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions (Smith and Read 1997). A number of studies have demonstrated that the AM symbiosis can protect the host plants against the detrimental effects of drought stress (Augé 2001, 2004; Ruiz-Lozano 2003). These studies have suggested several mechanisms by which the AM symbiosis can alleviate drought stress in host plants. The most important are: direct uptake and transfer of water through the fungal hyphae to the host plant (Hardie 1985; Ruiz-Lozano and Azcón 1995; Marulanda et al. 2003), better osmotic adjustment of AM plants (Augé et al. 1992; Ruiz-Lozano et al. 1995; Kubikova et al.

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2001), enhancement of plant gas exchange (Augé et al. 1992; Ruiz-Lozano et al. 1995; Goicoechea at al. 1997; Green et al. 1998), changes in soil water retention properties (Augé et al. 2001), and protection against the oxidative damage generated by drought (Ruiz-Lozano et al. 1996, 2001; Porcel et al. 2003; Porcel and Ruiz-Lozano 2004).

The understanding of the water relations of AM plants and the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation has increased in recent years. However, there are still many unknown aspects which must be elucidated, mainly at the molecular level (Ruiz-Lozano 2003). For that reason, our research group has initiated an investigation aimed at evaluating the possible participation of drought-induced genes in the enhanced tolerance of AM plants to drought stress. The following sections present a discussion on the most important results obtained.

2 Late Embryogenesis Abundant Proteins

These are a group of proteins that accumulate in plant seeds during their maturation phase, when tolerance to desiccation is required (Close 1996). It has been demonstrated that late embryogenesis abundant (LEA) proteins also accumulate in vegetative plant tissues during periods of water deficit, which reinforced a role for these proteins as desiccation protectant (Moons et al. 1997). It seems that during cellular dehydration LEA proteins play an important role in maintenance of the structure of other proteins, vesicles or endomembrane structures in the sequestration of ions such as calcium, in binding or replacement of water, and functioning as molecular chaperones (Close 1996; Koag et al. 2003). The overexpression of LEA proteins in plants and yeast confers tolerance to osmotic stresses (Imai et al. 1996; Xu et al. 1996; Babu et al. 2004). Dehydrins are an important group of LEA proteins (LEA group 2). They represent the most conspicuous soluble proteins induced by a dehydration stress and have been observed in over 100 independent studies of drought stress, cold acclimation, salinity stress, embryo development and responses to ABA. Among the water-stress-induced proteins so far identified, dehydrins are the most frequently observed (Close 1997; Cellier et al. 1998). Dehydrins could play a fundamental role in the dehydration response of plants to a range of environmental and developmental stimuli (Close 1996). However, the existence of multiple targets for dehydrins (euchromatin, cytosol, cytoskeleton) suggest that the direct consequences of dehydrin activity are biochemically diverse.

As these proteins seems to be part of the universal plants responses against dehydration, it is of interest to determine whether the AM symbiosis is able to alter the pattern of dehydrin accumulation under drought stress and whether such possible alteration functions in the protection of the host plants against drought. As a first approach, we cloned two dehydrin-encoding genes from *Glycine max* (*gmlea 8* and *gmlea 10*) and one from *Lactuca sativa* (*lslea 1*) and analyzed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

The genes *gmlea* and *lslea* were only expressed in drought stressed treatments (Figs. 1 and 2), corroborating that these dehydrins are important for the plant response against drought stress (Cellier et al. 1998; Giordani et al. 1999). In lettuce plants, *lslea 1* gene was also induced by drought stress in the three treatments

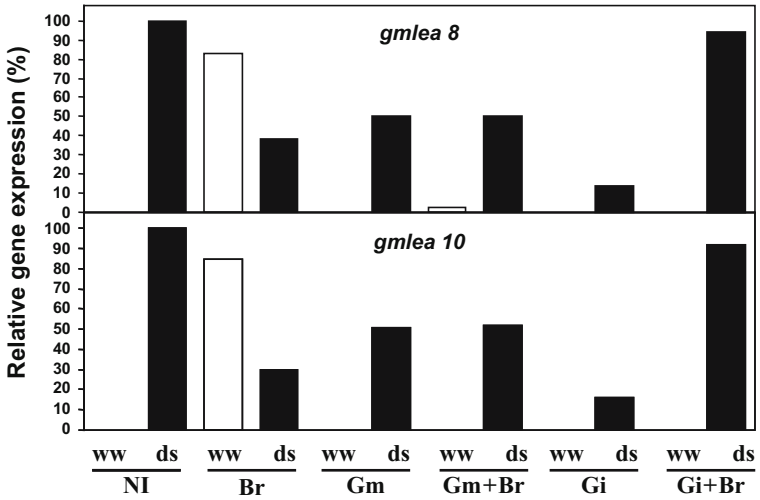

Fig. 1 Relative expression in soybean roots of *gmlea8* and *gmlea10* genes. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into each membrane. Treatments are designed as *NI* noninoculated controls, *Br Bradyrhizobium japonicum*, *Gm Glomus moosseae*, *Gm+Br G. mosseae* plus *B. japonicum*, *Gi Glomus intraradices*, *Gi+Br G. intraradices* plus *B. japonicum*. Plants were either well-watered (*ww*) or drought stressed (*ds*) for 10 days. Reproduced from Porcel et al. (2005b), with permission from Oxford University Press

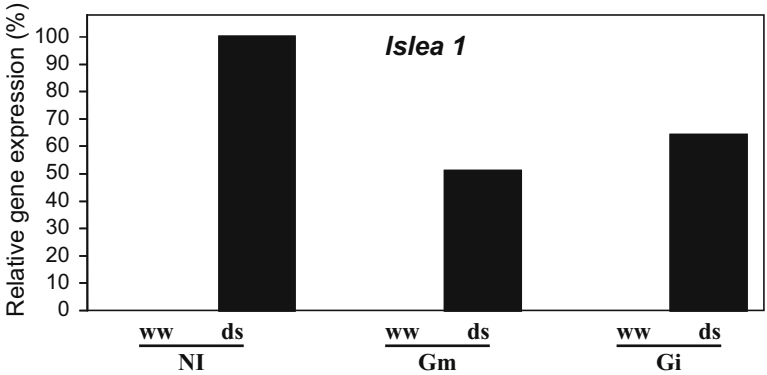

Fig. 2 Relative expression in lettuce roots of *lslea 1* gene. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into the membrane. Treatments are designed as *NI* noninoculated controls, *Gm Glomus moosseae*, *Gi Glomus intraradices*. Plants were either well-watered (*ww*) or drought stressed (*ds*) for 10 days. Reproduced from Porcel et al. (2005b), with permission from Oxford University Press

assayed (Fig. 2). However, the level of induction was clearly higher in roots from noninoculated plants than in roots from both AM treatments.

Another effect observed in the case of soybean (but not of lettuce) was the lower *gmlea* gene expression in roots from plants colonized by *G. intraradices* alone compared to that of plants colonized by *G. mosseae* alone (Fig. 1). However, functional diversity among different AM fungi has been widely observed in several aspects of the symbiosis (Burleigh et al. 2002).

In any case, the expression of *gmlea* and *lslea* genes in soybean and lettuce plants, respectively, was lower in drought stressed AM plants than in noninoculated plants. To understand this effect, it must be considered that abscisic acid (ABA) induces the expression of water deficit-responsive genes such as *lea* (Giordani et al. 1999). It has been proposed that mycorrhization can alter the levels of ABA in the host plant and that under drought stress the levels of ABA are lower in AM than in non-AM plants (Goicoechea et al. 1997; Estrada-Luna and Davis 2003); thus, the level of *lea* gene expression can be lower in these plants. Additionally, AM plants can be less strained by drought stress than non-AM plants and, for that reason, the expression of the *lea* genes studied is lower. In previous studies in which other authors and ourselves have found physiological or biochemical mechanisms involved in the enhanced tolerance to drought stress in AM plants, it has been proposed that primary drought-avoidance mechanisms (i.e., direct water uptake by hyphae) or increased water uptake related to mycorrhizal changes in root morphology (Kothari et al. 1990) or soil structure (Augé et al. 2001; Augé 2004) might have contributed to the AM protection of host plants against drought (Porcel et al. 2003). This hypothesis was supported by data on relative water content (RWC), which were significantly higher in AM plants than in non-AM plants. Also, previous studies with soybean plants subjected to a similar drought stress level have shown that AM plants exhibit higher leaf water potential (ψ) than non-AM plants (Porcel and Ruiz-Lozano 2004).

Concluding, results demonstrate that the levels of *lea* transcript accumulation in soybean and lettuce plants colonized by either *G. mosseae* or *G. intraradices* were considerably lower than those of the corresponding nonmycorrhizal plants, suggesting that the accumulation of LEA proteins is not a mechanism by which the AM symbiosis protects their host plant (Porcel et al. 2005b). This rather suggests that mycorrhizal plants were less strained by drought due to primary drought-avoidance mechanisms.

3 Δ^1-Pyrroline-5-Carboxylate Synthetase (P_5CS)

As a soil dries out and its water potential becomes more negative, plants must decrease their water potential to maintain a favorable water flow gradient from soil into roots. The most important mechanism to achieve such an effect, known as osmotic adjustment or osmoregulation, is to decrease the plant osmotic potential by active accumulation of organic ions or solutes (Morgan 1984). Of these metabolites, proline is probably the most widespread in plants (Yoshiba et al. 1995;

Armengaud et al. 2004). It has been shown that proline accumulates under conditions of water shortage, high salinity, chilling, heat, and heavy metal exposure. It plays a major role in osmoregulation and osmotolerance (Demir 2000). However, proline performs also an important function as a protective compatible osmolyte in scavenging of free radicals and facilitating a correction of altered redox potential by replenishment of the NADP+ supply (Hasegawa et al. 2000; Hare et al. 2003).

Accumulation of proline is mainly due to de novo synthesis, although a reduced rate of catabolism has also been observed (Kishor et al. 1995). The first two steps of proline biosynthesis are catalyzed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) by means of its γ-glutamil kinase and glutamic-γ-semialdehyde dehydrogenase activities. Subsequently, the Δ^1-pyrroline-5-carboxylate (P5C) formed is reduced by P5C reductase (P5CR) to proline (Hu et al. 1992). The rate-limiting step in this pathway is represented by the γ-glutamil kinase activity of P5CS, which is sensitive to feedback inhibition by relatively low levels of proline (Smith et al. 1984). In addition, in *Arabidopsis*, the P5CS-encoding gene is induced by drought stress, salinity and ABA, but P5CR is not (Yoshiba et al. 1995). The overexpression of the P5CS-encoding gene in transgenic tobacco plants has been shown to increase proline production and to confer tolerance of such plants to osmotic stress (Kishor et al. 1995). Hence, the P5CS-encoding gene is of key importance for the biosynthesis of proline in plants (Ábrahám et al. 2003).

Investigations carried out so far on osmoregulation in the AM symbiosis are scarce and somewhat contradictory. While some studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought (Ruiz-Lozano et al. 1995; Azcón et al. 1996; Goicoechea et al. 1998), the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved. For instance, while plants colonized by *G. deserticola* accumulated 120 nmol of proline per g fresh weight, plants colonized by *G. intraradices* only accumulated 41 nmol proline per g fresh weight (Ruiz-Lozano et al. 1995). It has also been shown that under low Ca in the medium AM plants accumulated more proline than nonAM plants when subjected to PEG-induced drought stress, while under high Ca in the medium this was not so (Ruiz-Lozano and Azcón 1997). On the contrary, other studies regarding drought (Ramakrishnan et al. 1988) or salt stress (Ruiz-Lozano et al. 1996) have shown a lower proline accumulation in AM plants than in nonAM ones.

Determining the expression pattern of genes such as *p5cs* in AM plants under osmotic stress conditions should provide an insight into the role of the AM symbiosis in the process of osmotic adjustment during drought stress. We cloned a P5CS-encoding gene from *Glycine max* (*gmp5cs*) and another from *Lactuca sativa* (*lsp5cs*) and analyzed their contribution to the response against drought in mycorrhizal soybean and lettuce plants. In fact, several investigations on the relationship between the expression of the key gene involved in the synthesis of proline (*p5cs*) and the accumulation of proline under water stress indicate that the level of proline in plants is mainly regulated at the transcriptional level during water stress (Hu et al. 1992; Yoshiba et al. 1995; Ábrahám et al. 2003; Armengaud et al. 2004).

Results showed that *gmp5cs* and *lsp5cs* (Figs. 3a and b, 4) genes responded to drought and were upregulated in drought-stressed treatments, suggesting that they

are important for the plant response against water deficit (Kishor et al. 1995; Hare et al. 2003; Parvanova et al. 2004). A contrasting result was obtained, however, in soybean plants singly inoculated with *B. japonicum*, where the *gmp5cs* gene showed little up-regulation in roots under drought stressed conditions (Fig. 3a). To explain this result, it must be considered that the expression of *p5cs* genes has two regulatory pathways, an ABA-dependent and an ABA-independent pathway, and that both can act simultaneously and with cumulative effects (Savoure et al. 1997; Ábrahám et al. 2003). Hence, it may be possible that nodulation itself can be affecting one of these regulatory pathways, avoiding the accumulation of *p5cs* transcripts. In contrast, the mycorrhization of nodulated plants restore, at least in part, the normal *p5cs* transcripts accumulation pattern by compensating in some way such ABA-dependent and ABA-independent pathways.

In contrast, in nodules of plants singly inoculated with *B. japonicum*, the pattern of *gmp5cs* gene expression was the expected one (Fig. 3b), namely upregulation under drought stress conditions. An elevated rate of proline biosynthesis in nodules has been suggested to stimulate ureide synthesis in legumes and to help transfer redox potential from the nodule cytoplasm to the bacteroids (Kohl et al. 1988). Proline may also act as a carbon and nitrogen source for the bacteroids. An additional an

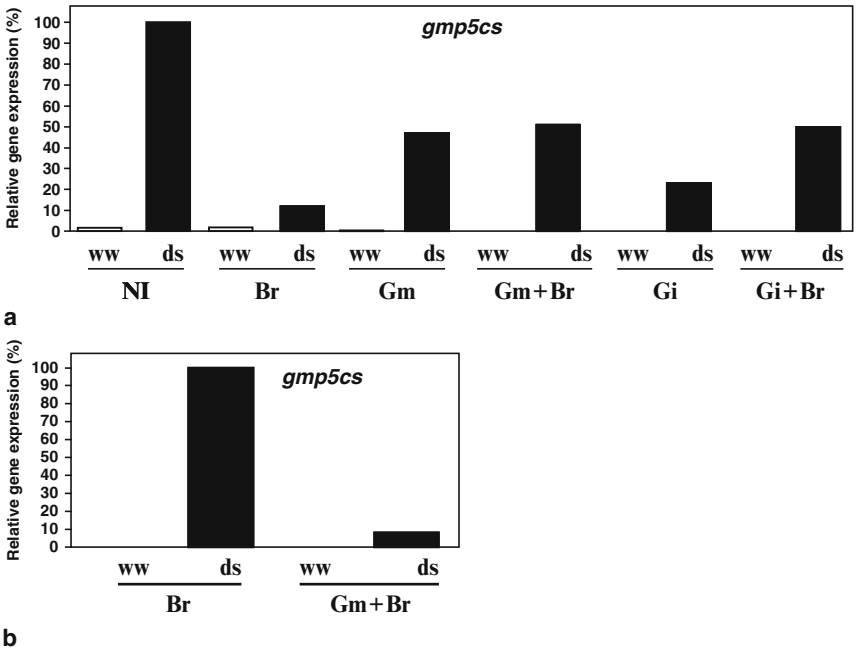

Fig. 3 Relative expression in soybean roots (**a**) and nodules (**b**) of *gmp5cs* gene. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into each membrane. Treatments are designed as in Fig. 1. Reproduced from Porcel et al. (2004), with permission from Elsevier

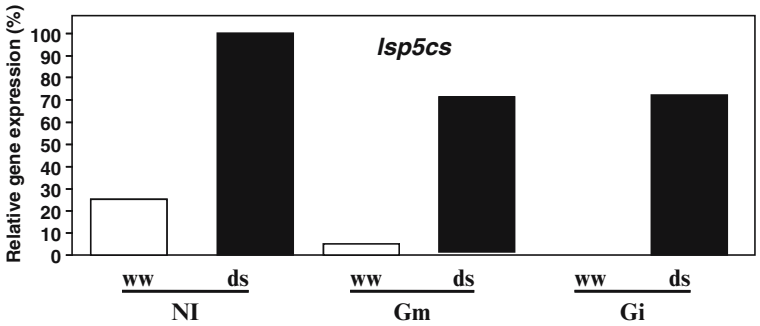

Fig. 4 Relative expression in lettuce roots of *lsp5cs* gene. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into each membrane. Treatments are designed as in Fig. 2. Reproduced from Porcel et al. (2004), with permission from Elsevier

important role of proline in nodules may be its involvement in osmoregulation (Delauney and Verma 1993). In fact, the osmoticum in infected nodule cells is known to be 4- to 5-fold higher than in root cells (Verma et al. 1978). Hence, the upregulation of *gmp5cs* in nodules of droughted plants may represent an osmoregulatory adaptation to increased concentration of solutes. What is not explained by that hypothesis is why the expression of the *gmp5cs* gene in nodules from soybean plants dually inoculated with the *G. mosseae* and with *B. japonicum* was considerably lower than in the corresponding nonmycorrhizal plants. However, under drought stress, AM plants normally present lower levels of ABA (Goicoechea et al. 1997; Estrada-Luna and Davies 2003). Hence, an ABA-dependent regulation pathway could explain the decrease in *gmp5cs gene* expression in nodules of these double inoculated plants as compared to those of single nodulated soybean plants. This mechanism may explain, in the same way, why the levels of *gmp5cs* and *lsp5cs* gene expression are lower in roots from droughted soybean and lettuce AM plants than in roots from droughted soybean and lettuce noninoculated plants.

Nevertheless, as also happened with *lea* genes, the expression of *gmp5cs* and *lslp5cs* genes decreased in drought stressed AM plants as compared to noninoculated plants (Figs. 3a and b, 4). This was probably due to a decrease in ABA level in AM plants and to the fact that AM plants were less strained by drought stress than nonAM plants by primary drought-avoidance mechanisms. The results demonstrate that the induction of *p5cs* gene do not seems to be a mechanism by which the AM symbiosis protects their host plant (Porcel et al. 2004).

4 Genes Encoding 14-3-3 Proteins and Binding Proteins

14-3-3 proteins are ubiquitous eukaryotic proteins that have wide-ranging regulatory functions by acting as phosphoserine/phosphothreonine-binding proteins (Roberts and de Bruxelles 2002). These proteins function in the regulation of signal

transduction pathways, generally acting as adapters, chaperones, activators or repressors (Palmgren et al. 1998), and they regulate the activities of a wide array of targets via direct protein–protein interactions. Binding of 14-3-3 proteins to a target serves either to directly regulate the activity of that protein, to affect its interactions with other protein or to modify the intracellular localization of the target (Roberts 2003). Targets for 14-3-3 include proteins involved in metabolism, signal transduction, chromatin function, ion transport, and vesicle trafficking (Roberts 2003).

14-3-3 protein family plays a central role in stress resistance, disease and growth control during the cell life-cycle (Chung et al. 1999). In the case of stress responses, support for such roles comes from the observation of changes in 14-3-3 gene expression during stress responses and from the detection of interactions between 14-3-3s and proteins with signaling or protective functions (Roberts et al. 2002; Wang et al. 2003).

The luminal binding protein (BiP) is a molecular chaperone of endoplasmic reticulum (ER) present in all kingdoms. The role of BiP in the ER is to transiently bind to unfolded proteins and to prevent intramolecular and intermolecular interactions that can result in permanent misfolding or aggregation, with the subsequent loss of their function (Gething and Sambrook 1992; Hendershot et al. 1996). Thus, both the increase of secretory activity and accumulation of unfolded proteins within the ER, as usually happens under abiotic stresses, result in the induction of BiP (Galili et al. 1998).

Some studies have demonstrated that overexpression of BiP genes in cultured mammalian cells and tobacco leaf protoplast attenuates ER stress caused by ionophore or tunicamycin (Laitusis et al. 1999). It is also well known that overexpression of BiP in mammalian cultured cells (Laitusis et al. 1999) prevents the induction of unfolded protein response (UPR)-induced genes and increases cell tolerance to stress, suggesting that BiP directly alleviates the ER stress. Plant BiP expression has been shown to respond to a variety of abiotic and biotic stress conditions, such as water stress, fungus infestation, insect attack, nutritional stress, cold acclimation and elicitors of the plant-pathogenesis response (Anderson et al. 1994; Kalinski et al. 1995; Figueiredo et al. 1997; Fontes et al. 1999). Furthermore, it has been demonstrated that constitutive overexpression of BiP in tobacco is enough to confer tolerance to water stress (Alvim et al. 2001).

Many studies on 14-3-3 proteins or BiPs have been carried out in plants, animals and yeasts. In contrast, there is no information about these proteins with chaperone activity in AM fungi or in the AM symbiosis. A 14-3-3 protein- and a BiP-encoding genes from *Glomus intraradices* were identified after differential hybridization of a cDNA library constructed from the fungus growing in vitro and subjected to drought stress by addition of 25% PEG 6000. Subsequently, their expression patterns were studied under drought stress in vitro and also when forming natural symbioses with different host plants.

The up-regulation of *Gi14-3-3* (Fig. 5) and *GiBiP* (Fig. 6) genes under conditions of water deficit (induced in vitro by PEG addition or in vivo by withholding plant irrigation) indicates that these fungal genes have a role in the answer of the fungus against drought. These genes are, probably, involved in the protection of

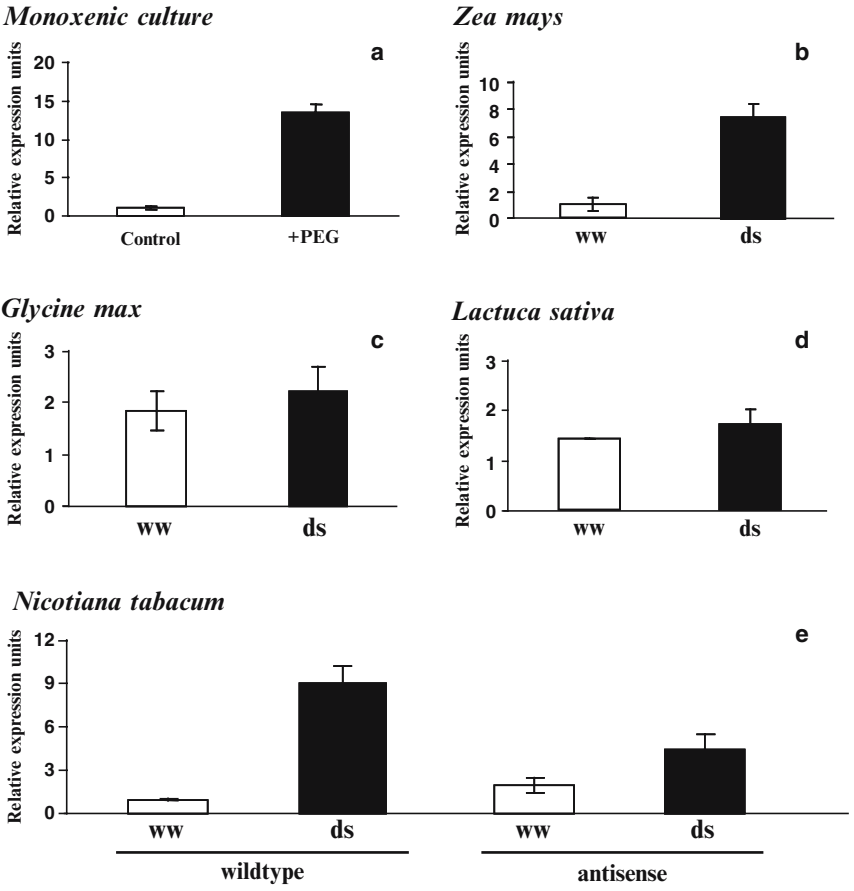

Fig. 5 Analysis of *Gi14-3-3* gene expression by real time quantitative RT-PCR in the AM fungus *G. intraradices* grown (**a**) in vitro and subjected to drought by addition of PEG (25%) to the growing medium or maintained under control conditions without PEG, or in the AM fungus *G. intraradices* during natural symbioses with maize plants (**b**), soybean plants (**c**), lettuce plants (**d**) or two tobacco plant lines (**e**), an aquaporin antisense mutant and the corresponding wild type. Plants were either well-watered (*ww*) or drought stressed (*ds*). Reproduced from Porcel et al. (2006b), with permission from Springer

the fungus itself (induction of the gene in vitro) and may be also involved in the protection of the host plant (induction of gene expression when forming natural symbiosis with plants).

It has been demonstrated that one of the effects of 14-3-3 proteins against osmotic stresses is carried out through the activation of the plasma membrane proton ATPase (Chelysheva et al. 1999; Babakov et al. 2000; Kerkeb et al. 2002). The activity of plasma membrane H^+-ATPase is highly regulated by factors that affect the cell physiology, including stress conditions (Palmgren 1998), and enhanced ATPase

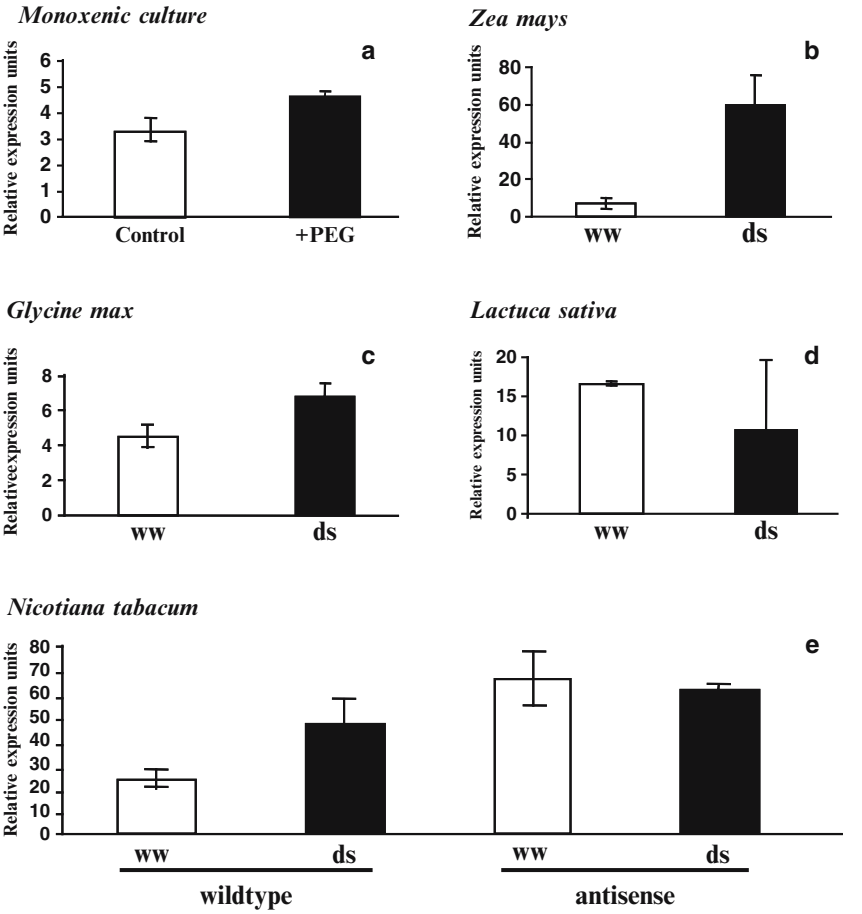

Fig. 6 Analysis of *GiBiP* gene expression by real time quantitative RT-PCR in the AM fungus *G. intraradices* grown (**a**) in vitro and subjected to drought by addition of PEG (25%) to the growing medium or maintained under control conditions without PEG, or in the AM fungus *G. intraradices* during natural symbioses with maize plants (**b**), soybean plants (**c**), lettuce plants (**d**) or two tobacco plant lines (**e**), an aquaporin antisense mutant and the corresponding wild type. Plants were either well-watered (*ww*) or drought stressed (*ds*). Reproduced from Porcel et al. (2007), with permission from Elsevier

activity is crucial for the protective system that different organisms have developed against external adverse influences (Serrano 1989). In the case of plants, the effects of 14-3-3 proteins are important for tolerance to water limitation since it has been shown that the plasma membrane H^+-ATPase plays an essential role in the regulation of plant cell turgor. In fact, it exports protons to create an electrochemical gradient across the plasma membrane, which is then used by cell as the driving force for nutrient uptake, phloem loading, water movement, stomatal closure and opening (Comparot et al. 2003). There is clear evidence for 14-3-3 mediated activation of the

H^+-ATPase in response to osmotic stresses. It has been demonstrated that osmotic stress induces a redistribution of 14-3-3 proteins between the cytoplasm and the plasma membrane of sugar beet cells. This effect is accompanied by an increase in H^+-pump activity (Chelysheva et al. 1999; Babakov et al. 2000; Kerkeb et al. 2002). Increased H^+ transport through phosphorylation and 14-3-3 binding to the proton ATPase are part of the early responses of cells to perturbation in growth conditions such as osmotic stress (Finnie et al. 2002; Kerkeb et al. 2002).

The induction of BiP mRNA by osmotic stress may represent a primary response to water stress that is activated as soon as the stress is sensed and may accommodate a regulatory function. In fact, BiP has been shown to associate with water-stress-induced proteins (Cascardo et al. 2000). The protective role of BiP against water stress may be the preservation of protein structure and of high secretory activity mediated by the water stress adaptive cellular response (Ingram and Bartels 1996).

Curiously, a non-significant effect of drought stress on *Gi14-3-3* gene expression was observed in this study when the fungus was associated to soybean plants (Fig. 5c). This result may be related to the fact that the AM colonization in these plants was considerably lower than in the rest of treatments. In any case, the induction of *Gi14-3-3* or *GiBiP* genes was more or less intense depending on the host plant (Figs. 5 and 6). This was quite evident when the fungus was associated to the two plants that are more sensitive to drought (lettuce and antisense tobacco plants) (Ruiz-Lozano et al. 1995; Porcel et al. 2005a). The varying results obtained with the different plants assayed indicate that the importance of *Gi14-3-3* and *GiBiP* when coping with drought stress may depend on the intrinsic physiological characteristics of the host plant. Hence, results suggest that Gi14-3-3 and GiBiP proteins take part of the mechanisms by which the AM symbiosis enhances the tolerance of the host plant against drought, although the real implication of these proteins may depend on the sensitivity of the host plant against water deficit (Porcel et al. 2006b, 2007).

Our findings provide new evidence that the contribution of AM fungi to the enhanced drought tolerance of the host plant can be mediated by proteins with chaperone-like activity, such as 14-3-3 or BiP proteins. However, as 14-3-3 and BiP proteins have multiple targets in the cell, the precise mechanism of Gi14-3-3- or GiBiP-mediated drought stress tolerance remains unknown. It is likely that Gi14-3-3 protein can regulate the activity of plasma membrane H+-ATPases of either the fungus or the host plant, in order to activate its pumping activity, which is essential to cope with osmotic stress (Serrano 1989; Palmgren 1998; Comparot et al. 2003), while GiBiP protein may facilitate the proper folding and maturation of water stress-induced proteins involved in the osmotic response mechanism (Cascardo et al. 2000).

5 Modulation of Aquaporins

Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient across membranes of living cells. These proteins belong to the large major intrinsic protein (MIP) family of

transmembrane proteins and are represented in all kingdoms (Maurel 1997). Two major classes of plant aquaporins, located in the plasma membrane (PIPs) or tonoplast (TIPs), respectively, have been identified so far. Another two classes of plant aquaporins are the homologues to the soybean Nodulin-26 aquaporin (NIPs) and the small basic intrinsic proteins (SIPs) (Johanson et al. 2001). The localization and function of SIPs are unknown at the moment (Luu and Maurel 2005), although the membrane of endoplasmic reticulum seems to contain SIPs (Ishikawa et al. 2005).

The discovery of aquaporins in plants has caused a significant change in the understanding of plant–water relations. In recent years, much effort has been concentrated on investigating the function and regulation of PIP aquaporins. These aquaporins seem to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots where they mediate most of soil water uptake (Javot and Maurel 2002), and transgenic plants downregulating one or more PIP genes had lower root water uptake capacity (Siefritz et al. 2002; Javot et al. 2003). However, the relationship that exists between aquaporins and plant responses to drought still remains elusive and with contradictory results (Aharon et al. 2003; Lian et al. 2004). Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in AM plants had never been investigated. Krajinski et al. (2000) proposed that the upregulation of aquaporins by the AM symbiosis probably optimizes nutrient and water exchange between both symbiotic partners. They may also permit efficient osmoregulation of the highly compartmented root cells (Maurel et al. 2002). However, the studies by Krajinski et al. (2000) were carried out under well-watered conditions and they did not test the expression of the aquaporin gene in AM plants under drought stress conditions. Several aquaporin-encoding genes have been shown to be upregulated in ectomycorrhizal poplar plants, and this was correlated with an increased water transport capacity of mycorrhizal poplar roots (Marjanovic et al. 2005). Finally, Porcel et al. (2005a) have shown that the impairment of a PIP gene in an antisense tobacco mutant reduced the symbiotic efficiency of two AM fungi under drought stress conditions.

Tolerance to drought stress in plants is a complex phenomenon and involves many changes at both biochemical and physiological levels (Ingram and Bartels 1996). Osmotic adjustment and modulation of tissue hydraulic conductivity are both required to maintain tissue water potential (Bohnert et al. 1995). Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurel 1997). Since aquaporins are regulated both at transcriptional and activity levels (Martre et al. 2002), we have considered it of interest to study whether the expression of aquaporin-encoding genes in roots is altered by the AM symbiosis as a mechanisms to enhance host plant tolerance to water deficit. To achieve this, genes encoding plasma membrane aquaporins (PIPs) from soybean and lettuce were cloned and their expression pattern studied, in AM and non-AM plants cultivated under well-watered or drought stress conditions. If AM fungi can transfer water to the root of the host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be upregulated in order to allow a higher rate of transcellular water flow (Javot and Maurel 2002).

Results showed that, in contrast to the above hypothesis, the PIP genes studied were downregulated both in soybean (Fig. 7) and lettuce (Fig. 8) under drought stress and that such downregulation was even more severe in plants colonized by *G. mosseae* than in non-AM plants. A similar result was obtained very recently by Ouziad et al. (2006) regarding the expression of PIP and TIP genes in roots of AM tomato plants subjected to salt stress. Furthermore, when the expression of *gmPIP2* was analyzed in a time-course, we observed that AM plants downregulated that gene significantly at 5 and 12 days after inoculation, while both non-AM control plants maintained *gmPIP2* gene expression almost unaltered until 20 days after inoculation (data not shown). This effect of the AM symbiosis anticipating the downregulation of *gmPIP2* gene may have a physiological importance to help AM plants to cope with drought stress (Porcel et al. 2006a). In fact, according to Aharon et al. (2003), the overexpression of a PIP aquaporin in transgenic tobacco improves plant vigour under favorable growth conditions, but the overexpression of such PIP gene has no beneficial effect under salt stress, and even has a negative effect during drought stress, causing fast wilting. Hence, the decreased expression of plasma

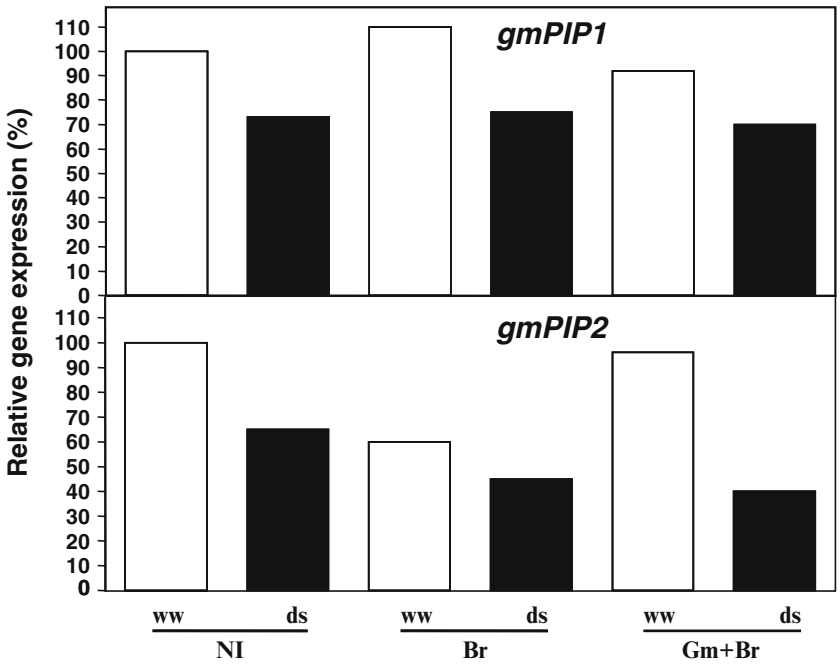

Fig. 7 Relative expression in soybean roots of *gmPIP1* and *gmPIP2* genes. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into each membrane. Treatments are designed as in Fig. 1. Reproduced from Porcel et al. (2006a), with permission from Springer

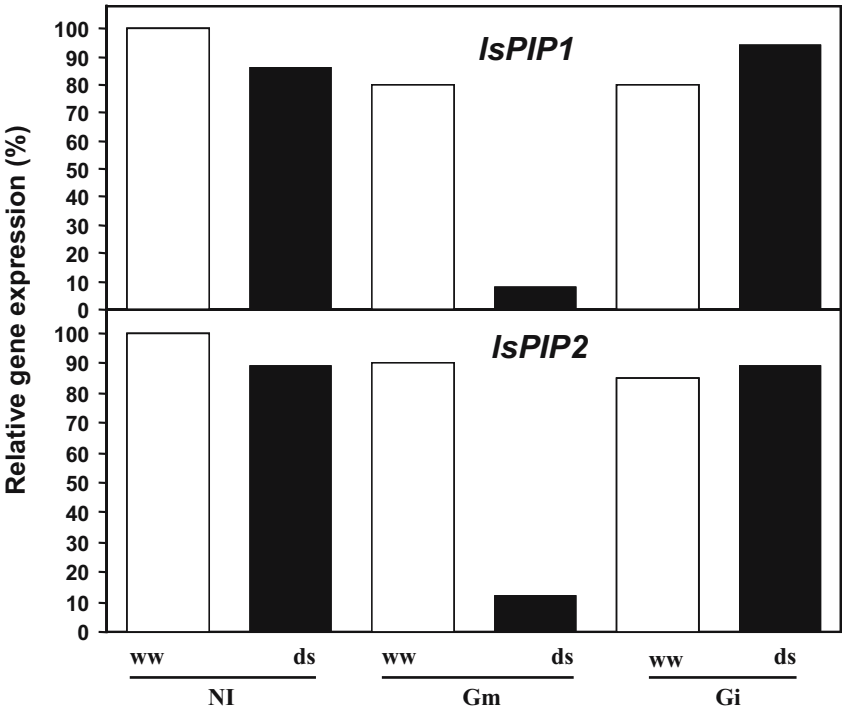

Fig. 8 Relative expression in lettuce roots of *lsPIP1* and *lsPIP2* genes. Values were obtained after normalization of northern blots to the ribosomal RNA loaded into each membrane. Treatments are designed as in Fig. 2. Reproduced from Porcel et al. (2006a), with permission from Springer

membrane aquaporin genes during drought stress in AM plants can be a regulatory mechanism to limit the water lost from the cells (Barrieu et al. 1999). In support of this hypothesis data on leaf ψ and RWC show that AM plants (soybean and lettuce) had higher leaf ψ and water content than non-AM plants.

The up- or downregulation by drought stress of mRNAs encoding aquaporins homologues has been described in the roots of many plant species (Javot and Maurel 2002). There are currently two opposite descriptions of the role of aquaporins in response to dehydration stress (Smart et al. 2001). The first is based on evidence that expression of some aquaporins is induced under dehydration stress (Barrieu et al. 1999; Jang et al. 2004), which is predicted to result in greater membrane water permeability and facilitated water transport. The second is based on the fact that aquaporin activity is downregulated under dehydration stress, which should result in decreased membrane water permeability and may allow cellular water conservation (Yamada et al. 1995; Smart et al. 2001) during periods of dehydration stress.

Results from lettuce plants also colonized by *G. mosseae* point in the same direction (Fig. 8), namely that under drought stress conditions there is a higher downregulation of the PIP genes studied (and also at the protein level, as revealed

by western blot) in AM plants than in non-AM plants. In contrast to *G. mosseae*, plants colonized by *G. intraradices* do not exhibit such downregulation of PIP gene expression or protein accumulation. The expression of PIP genes under drought stress in these plants is similar to control non-AM plants. The exact reason for the different influence of *G. mosseae* and *G. intraradices* on lettuce *PIP* gene expression is not known. However, in a previous study, also with lettuce, we evaluated the ability of six AM fungal species, including *G. mosseae* and *G. intraradices*, to enhance the amount of soil water uptake by these plants (Marulanda et al. 2003). The study demonstrated that there were substantial differences among the six AM fungi used. One of the most efficient fungi stimulating water uptake by plants was *G. intraradices*, while *G. mosseae* showed a reduced ability to improve plant water uptake. This may suggest that the strategy of both fungi to protect the host plant against water deficit is different. *G. intraradices* seems to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that the water movement in these roots must be enhanced and, thus, the root water permeability must also increase, maybe by maintaining high levels of PIP aquaporin gene expression as we observe in this study. Contrarily, *G. mosseae* seems to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant and by that reason downregulates the expression of PIP genes. Such downregulation of PIP genes has been interpreted as a mechanism to decrease membrane water permeability and to allow cellular water conservation (Yamada et al. 1995; Smart et al. 2001). In any case, both strategies seems to protect the host plant in a similar way since lettuce plants had similar RWC and leaf ψ regardless of the fungus colonizing their roots.

Concluding, results suggest that AM plants respond to drought stress by downregulating the expression of the two *PIP* genes studied and anticipating its downregulation as compared to non-AM plants, rather than by maintaining high levels of these PIP genes expression (Porcel et al. 2006a). This downregulation of *PIP* genes is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservation. It must be considered, however, that as *PIP* are members of a multi-gene family, other *PIP* isoforms in soybean and lettuce plants may be regulated differently and that depending on the AM fungus implicated in the symbiosis the patter of aquaporin gene expression can also be different.

6 Conclusions

Considering the overall results presented in this review, it is evident that there are some aspects that are more promising than others for future research on that topic. It seems that there is no sense in investigating more in deep the possible role of *lea* and *p5cs* genes. This must be considered, however, with caution since we only analyzed a few of the *lea* genes belonging to the dehydrin group, while other *lea* genes existing in plants still remains to be checked. In contrast, the evidence obtained with these two genes suggest that it is of interest to study the possible role

of ABA in the modulation of the host plant answer to water deficit by AM symbiosis. In the same way, results obtained with aquaporin genes suggest the interest in studying the correlation between up- or downregulation of aquaporin gene expression and root hydraulic conductivity and plant water status. The number of aquaporin genes analyzed should also be enhanced since aquaporins constitute a multigenic family in plants and it is likely that some of the genes not analyzed so far can be regulated by the AM symbiosis in relation to the alleviation of drought stress in the host plant. Finally, the interesting results obtained in relation to *Gi14-3-3* and *GiBiP* genes, whose function can be exerted over a wide range of targets proteins, open an interesting perspective to identify final effectors proteins responsible of the enhanced tolerance against water deficit mediated by these 14-3-3 and BiP proteins.

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References

Ábrahám E, Rigó G, Székely G, Nagy R, Koncz C, Szabados L (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis*. Plant Mol Biol 51:363–372

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G (2003) Overexpression of a plasma membrane aquaporins in transgenic tobacco improves plant vigour under favourable growth conditions but not under drought or salt stress. Plant Cell 15:439–447

Alvim FC, Carolino SMB, Cascardo JCM, Nunes CC, Martínez CA, Otón WC, Fontes EPB (2001) Enhanced accumulation of BiP in transgenic plants confers tolerance to water stress. Plant Physiol 126:1042–1054

Anderson JV, Li QB, Haskell DW, Guy CL (1994) Structural organization of the spinach endoplasic reticulum luminal 70-kilodalton heat-shock cognate gene and expression of 70-kilodalton heat-shock genes during cold acclimation. Plant Physiol 104:1359–1370

Armengaud P, Thiery L, Buhot N, Grenier-De March G, Savoure A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant 120:442–50

Augé RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Augé RM (2004) Arbuscular mycorrhizae and soil/plant water relations. Can J Soil Sci 84:373–381.

Augé RM, Foster JG, Loescher WH, Stodola AW (1992) Symplastic sugar and free amino acid molality of Rosa roots with regard to mycorrhizal colonization and drought. Symbiosis 12:1–17

Augé RM, Stodola AW, Tims JE, Saxton AM (2001) Moisture retention properties of a mycorrhizal soil. Plant Soil 230:87–97

Azcón R, Gómez M, Tobar RM (1996) Physiological and nutritional responses by *Lactuca sativa* to nitrogen sources and mycorrhizal fungi under drought. Biol Fert Soils 22:156–161

Babakov AV, Chelysheva VV, Klychnikov OI, Zorinyanz SE, Trofimova MS, de Boer AH (2000) Involvement of 14-3-3 proteins in the osmotic regulation of H^+-ATPase in plant plasma membranes. Babu RC, Zhang J, Blum A, Ho THD, Wu R, Nguyen HT (2004) *HVA1*, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection. Plant Sci 166:855–862

Barrieu F, Marty-Mazars D, Thomas D, Chaumont F, Charbonnier M, Marty F (1999) Desiccation and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in cauliflower cells. Planta 209:77–86

Babakov AV, Chelysheva VV, Klychnikov OI, Zorinyanz SE, Trofimova MS, de Boer AH (2000) Involvement of 14-3-3 proteins in the osmotic regulation of H^+-ATPase in plant plasma membranes. Planta 211:446–448

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. Plant Cell 7:1099–1111

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2:48–54

Bray EA (2004) Genes commonly regulated by water-deficit stress in Arabidopsis thaliana. J Exp Bot 55:2331–2341

Burleigh SH, Cavagnaro T, Jakobsen I (2002) Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J Exp Bot 53:1593–1601

Cascardo JCM, Almeida RS, Buzeli RAA, Carolino SMB, Otoni WC, Fontes EPB (2000) The phosphorylation state and expression of soybean BiP isoforms are differentially regulated following abiotic stresses. J Biol Chem 275:14994–14500

Cellier F, Conéjéro G, Breitler JC, Casse F (1998) Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Plant Physiol 116:319–328

Chelysheva VV, Smolenskaya IN, Trofimova MC, Babakov AV, Muromtsev GS (1999) Role of the 14-3-3 proteins in the regulation of H^+-ATPase activity in the plasma membrane of suspension-cultured sugar beet cells under cold stress. FEBS Lett 456:22–26

Chung HJ, Sehnke PC, Ferl RJ (1999) The 14-3-3 proteins: cellular regulator of plant metabolism. Trends Plant Sci 4:367–371

Close TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97:795–803

Close TJ (1997) Dehydrins: a commonalty in the response of plants to dehydration and low temperature. Physiol Plant 100:291–296

Comparot S, Lingiah G, Martin T (2003) Function and specificity of 14-3-3 proteins in the regulation of carbohydrate and nitrogen metabolism. J Exp Bot 54:59–604

Delauney AJ, Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. Plant J 4:215–23.

Demir Y (2000) Growth and proline content of germinating wheat genotypes under ultraviolet light. Turk J Bot 24:67–70

Estrada-Luna AA, Davies FT (2003) Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated child and pepper (*Capsicum annuum*) plantlets during acclimatization and post-acclimatization. J Plant Physiol 160: 7073–1083

Figueiredo JEF, Cascardo JCM, Carolino SMB, Alvim FC, Fontes EPB (1997) Water-stress regulation and molecular analysis of the soybean BiP gene family. Braz J Plant Physiol 9:103–110

Finnie C, Andersen CH, Borch J, Gjetting S, Christensen AB, de Boer AH, Thordal-Christensen H, Collinge DB (2002) Do 14-3-3 proteins and plasma membrane H^+-ATPases interact in the barley epidermis in response to the barley powdery mildew fungus? Plant Mol Biol 49:137–147

Fontes MA, Otoni WC, Carolino SMB, Brommonshenkel SH, Fontes EPB, Fári M, Louro RP (1999) Hyperhydricity in pepper plants regenerated in, vitro: involvement of BiP (binding protein) and ultrastructural aspects. Plant Cell Rep 19:81–87

Galili G, Sengupta-Gopalam C, Ceriotti A (1998) The endoplasmic reticulum of plant cells and its role in protein maturation and biogenesis of oil bodies. Plant Mol Biol 38:1–29

Gething MJ, Sambrook J (1992) Protein folding in the cell. Nature 355:33–45

Giordani T, Natali L, D'Ercole A, Pugliesi C, Fambrini M, Vernieri P, Vitagliano C, Cavallini A (1999) Expression of a dehydrin gene during embryo development and drought stress in ABA-deficient mutants of sunflower (*Helianthus annuus* L). Plant Mol Biol 39:739–748

Goicoechea N, Antolin MC, Sánchez-Díaz M (1997) Gas exchange is related to the hormone balance in mycorrhizal or nitrogen-fixing alfalfa subjected to drought. Physiol Plant 100:989–997

Goicoechea N, Szalai G, Antolín MC, Sánchez-Díaz M, Paldi E (1998) Influence of arbuscular mycorrhizae and *Rhizobium* on free polyamines and proline levels in water-stressed alfalfa. J Plant Physiol 153:706–711

Green CD, Stodola A, Augé RM (1998) Transpiration of detached leaves from mycorrhizal and nonmycorrhizal cowpea and rose plants given varying abscisic acid, pH, calcium and phosphorus. Mycorrhiza 8:93–99

Hardie K (1985) The effect of removal of extraradical hyphae on water uptake by vesicular-arbuscular mycorrhizal plants. New Phytol 101:677–684

Hare PD, Cress WA, van Staden J (2003) A regulatory role for proline metabolism in stimulating *Arabidopsis thaliana* seed germination. Plant Growth Regul 39:41–50

Hasegawa PM, Bressan RA, Zhu, JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. An Rev Plant Physiol Plant Mol Biol 51:463–499

Hendershot L, Wei J, Gaut J, Melnick J, Aviel S, Argon Y (1996) Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. Proc Natl Acad Sci USA 93:5269–5274

Hu CA, Delaunew AJ, Verma DPS (1992) A bifunctional enzyme (Δ^1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA 89:9354–9358

Imai R, Chang L, Ohta A, Bray EA, Takagi M (1996) A *lea*-class gene of tomato confers salt and freezing tolerance when over expressed in *Saccharomyces cerevisisae*. Gene 170:243–248

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377–403

Ishikawa F, Suga S, Uemura T, Sato MH, Maeshima M (2005) Novel type aquaporin SIPs are mainly localized to the ER membrane and show cell-specific expression in *Arabidopsis thaliana*. FEBS Lett 579:5814–5820

Jang JY, Kim DG, Kim YO, Kim JS, Kang H (2004) An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. Plant Mol Biol 54:713–725

Javot H, Maurel C (2002) The role of aquaporins in root water uptake. Ann Bot 90:301–313

Javot H, Lauvergeat V, Santoni V, Martin-Laurent F, Guclu J, Vinh J, Heyes J, Franck KI, Schaffner AR, Bouchez D. Maurel C (2003) Role of a single aquaporin isoform in root water uptake. Plant Cell 15:509–522

Johanson U, Karlsson M, Johanson I, Gustavsson S, Sjövall S, Fraysse L, Weigh AR, Kjellbom P (2001) The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants. Plant Physiol 126:1358–1369

Kalinski A, Rowley DL, Loer DS, Foley C, Buta G, Herman E (1995) Binding protein expression is subject to temporal, developmental and stress induced regulation in terminally differentiated soybean organs. Planta 195:611–621

Kerkeb L, Vanema K, Donaire JP, Rodriguez-Rosales MP (2002) Enhanced H^+/ATP coupling ratio of H^+-ATPase and increased 14-3-3 protein content in plasma membrane of tomato cells upon osmotic shock. Physiol Plant 116:37–41

Kishor PB, Hong Z, Miao GH, Hu CA, Verma DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxilate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108:1387–1394

Koag MC, Fenton RD, Wilkens S, Close T (2003) The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity. Plant Physiol 131:309–316

Kohl DH, Schubert KR, Carter MB, Hagedorn CH, Shearer G (1988) Proline metabolism in N_2-fixing root nodules: energy transfer and regulation of purine synthesis. Proc Natl Acad Sci USA 87:2036–2040

Kothari SK, Marschner H, George E (1990) Effect of VA mycorrhizal fungi and rhizospere microorganisms on root and shoot morphology, growth and water relations in maize. New Phytol 116:303–311

Krajinski F, Biela A, Schubert D, Gianinazzi-Pearson V, Kaldenhoff R, Franken P (2000) Arbuscular mycorrhiza development regulates the mRNA abundance of *Mtaqp1* encoding a mercury-insensitive aquaporin of *Medicago truncatula*. Planta 211:85–90

Kramer PJ, Boyer JS (1997) Water relations of plants and soils. Academic, San Diego

Kubikova E, Moore JL, Ownlew BH, Mullen MD, Augé RM (2001) Mycorrhizal impact on osmotic adjustment in *Ocimum basilicum* during a lethal drying episode. J Plant Physiol 158:1227–1230

Laitusis AL, Brostrom MA, Brostrom CO (1999) The dynamic role of GRP78/BiP in the coordination of mRNA translation with protein processing. J Biol Chem 274:486–493

Lian HL, Yu X, Ye Q, Ding XS, Kitagawa Y, Swak SS, Su WA, Tang ZC (2004) The role of aquaporin RWC3 in drought avoidance in rice. Plant Cell Physiol 15:481–489

Luu DT, Maurel C (2005) Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ 28:85–96

Marjanovic Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weia M, Hampp R, Nehls U (2005) Aquaporins in poplar: what a difference a simbiont makes! Planta 222:258–268

Martre P, Morillon R, Barrieu F, North GB, Nobel PS, Chrispeels MJ (2002) Plasma membrane aquaporins play a significant role during recovery from water deficit. Plant Physiol 130:2101–2110

Marulanda A, Azcón R, Ruiz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* L. plants under drought stress. Physiol Plant 119:526–533.

Maurel C (1997) Aquaporins and water permeability of plant membranes. Annu Rev Plant Physiol Plant Mol Biol 48:399–429

Maurel C, Javot H, Lauvergeat V, Gerbeau P, Tournaire C, Santoni V, Heyes J (2002) Molecular physiology of aquaporins in plants. Int Rev Cytol 215:105–148

Moons A, De Keyser A, Van Montagu M (1997) A group 3 LEA cDNA of rice, responsive to abscisic acid, but no to jasmonic acid, shows variety-specific differences in salt stress response. Gene 191:197–204

Morgan JM (1984) Osmoregulation and water stress in higher plants. Annu Rev Plant Physiol 33:299–319

Ouziad F, Wilde P, Schmelzer E, Hildebrandt U, Bothe H (2006) Analysis of expression of aquaporins and Na+/H+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environ Exp Bot 57:177–186

Palmgren MG (1998) Proton gradients and plant growth: role of the plasma membrane H^+-ATPase. Adv Bot Res 28:1–70

Palmgren MG, Fuglsang AT, Jahn T (1998) Deciphering the role of 14-3-3 proteins. Exp Biol Online 3:1–17

Parvanova D, Ivanov S, Konstantinova T, Karanov E, Atanassov A, Tsvetkov, T, Alexieva V, Djilianov D (2004) Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiol Biochem 42:57–63

Porcel R, Ruiz-Lozano JM (2004) Arbuscular mycorrhizal influence on leaf water potential, solute accumulation and oxidative stress in soybean plants subjected to drought stress. J Exp Bot 55:1743–1750

Porcel R, Barea JM, Ruiz-Lozano JM (2003) Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. New Phytol 157:135–143

Porcel R, Azcón R, Ruiz-Lozano JM (2004) Evaluation of the role of genes encoding for Δ^1-pyrroline-5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. Physiol Mol Plant Pathol 65:211–221

Porcel R, Gómez M, Kaldenhoff R, Ruiz-Lozano JM (2005a) Impairment of *NtAQP1* gene expression in tobacco plants does not affect root colonization pattern by arbuscular mycorrhizal fungi but decreases their symbiotic efficiency under drought. Mycorrhiza 15:417–423

Porcel R, Azcón R, Ruiz-Lozano JM (2005b) Evaluation of the role of genes encoding for dehydrin proteins (LEA D-11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. J Exp Bot 56:1933–1942

Porcel R, Aroca R, Azcón R, Ruiz-Lozano JM (2006a) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. Plant Mol Biol 60:389–404

Porcel R, Aroca R, Cano C, Bago A, Ruiz-Lozano JM (2006b) Identification of a gene from the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* encoding for a 14-3-3 protein, that is up-regulated by drought stress during the AM symbiosis. Microb Ecol 52:575–582

Porcel R, Aroca R, Cano C, Bago A, Ruiz-Lozano JM (2007) A gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding a binding protein is up-regulated by drought stress during the mycorrhizal symbiosis. Environ Exp Bot 60:251–256

Ramakrishnan B, Johri BN, Gupta RK (1988) Influence of the VAM fungus *Glomus caledonius* on free proline accumulation in water-stressed maize. Cur Sci 57:1082–1084

Roberts MR (2003) 14-3-3 proteins find new partners in plant cell signalling. Trends Plant Sci 8:218–223

Roberts MR, de Bruxelles GL (2002) Plant 14-3-3 protein families: evidence for isoform-specific functions? Biochem Soc Trans 30:373–378

Roberts MR, Salinas J, Collinge DB (2002) 14-3-3 proteins and the response to abiotic and biotic stress. Plant Mol Biol 50:1031–1039

Ruiz-Lozano JM (2003) Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13:309–317.

Ruiz-Lozano JM, Azcón R (1995) Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. Physiol Plant 95:472–478

Ruiz-Lozano JM, Azcón R (1997) Effect of calcium application on the tolerance of mycorrhizal lettuce plants to polyethylene glycol-induced water stress. Symbiosis 23:9–22

Ruiz-Lozano JM, Azcón R, Gómez M (1995) Effects of arbuscular mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. Appl Environ Microbiol 61:456–460.

Ruiz-Lozano JM, Azcón R, Gómez M (1996) Alleviation of salt stress by arbuscular-mycorrhizal *Glomus* species in *Lactuca sativa* plants. Physiol Plant 98:767–772

Ruiz-Lozano JM, Collados C, Barea JM, Azcón R (2001) Arbuscular mycorrhizal symbiosis can alleviate drought-induced nodule senescence in soybean plants. New Phytol 151:493–502

Savoure A, Hua XJ, Bertauche N, Van Montagu M, Verbruggen N (1997) Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thalina*. Mol Gen Genet 254:104–109

Serrano R (1989) Structure and function of plasma membrane ATPase. Annu Rev Plant Physiol Plant Mol Biol 40:61–94

Siefritz F, Tyree MT, Lovisolo C, Schubert A, Kaldenhoff R (2002) PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. Plant Cell 14:869–876

Smart LB, Moskal WA, Cameron KD, Bennett AB (2001) Mip genes are down-regulated under drought stress in *Nicotiana glauca*. Plant Cell Physiol 42:686–693

Smith CJ, Deutch AH, Rushlow KE (1984) Purification and characteristics of a ψ-glutamyl kinase involved in *Escherichia coli* proline biosynthesis. J Bacteriol 157:545–551

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, San Diego

Verma DP, Kazazian V, Zogbi V, Bal AK (1978) Isolation and characterization of the membrane envelope enclosing the bacteroids in soybean root nodules. J Cell Biochem 78:919–936

Wang WX, Vinocur B, Altman A (2003) Plant response to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1–14

Xu DP, Duan X, Wang B, Hong B, Ho TD, Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110:249–257

Yamada S, Katsuhara M, Kelly WB, Michalowski CB, Bonhert HJ (1995) A family of transcripts encoding water channel proteins: tissue-specific expression in common ice plant. Plant Cell 7:1129–1142

Yoshiba Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Wada K, Harada Y, Shinozaki K (1995) Correlation between the induction of a gene for Δ^1-pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant J 7:751–760

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273.
Zhu JK, Hasegawa PM, Bressan R (1997) Molecular aspects of osmotic stress in plants. Crit Rev Plant Sci 16:253–277

The Beneficial Effect of Mycorrhizae on N Utilization by the Host-Plant: Myth or Reality?

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1 Introduction

Nitrogen represents the major element in plants. This element is present in most of the basic constituents: purines, pyrimidines, and amino acids, and is important for the autotrophic capacity of the plants, being part of the light harvesting molecules, e.g., chlorophyll. De facto, N deficiency in plants leads rapidly to chlorosis and stunted growth. Plants being sessile organisms, they are completely dependent on the N availability in the soil solution for their growth and productivity.

In soil, a harsh competition exists for N acquisition between the microorganisms (mainly bacteria and fungi) and the plants. However, plant species have developed various strategies of N acquisition. These strategies are including microorganism partners into a symbiotic association. First, legumes are associated with bacteria to assimilate aerial N. A second form of mutualism is the association between plants and mycorrhizal fungi. The fungus and the plant are associated underground forming a close bound between the hyphae and the root cells, the so-called mycorrhizal root.

These mycorrhizal roots are characterized by the presence of an extensive network of mycelium developing out of the root and exploring the soil. Therefore, the fungal partner considerably increases the volume of soil that is exploited compared to the nonmycorrhizal root system (Rousseau et al. 19994). The mycelium also develops between the root cells inside the root cortex. All mineral and nutrient exchanges between the host cell (carbohydrate release) and the fungal cells (phosphorus and nitrogen release) are thought to take place in this fungal–plant interface (Smith and Read 1997). Melin and Nilsson (1952) were the first to demonstrate the N uptake by the extra radical mycelium and its transfer to the host plant. Since this pioneering study, a considerable amount of work has been devoted to the capacities of mycorrhizal symbiosis to modify the access to the various N-forms from the soil, including the uptake and assimilation of N by the partners. The main purpose of

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this chapter is to summarize the present knowledge about the role of three main types of mycorrhizal associations made by ericoid, ectomycorrhizal and arbuscular mycorrhizal fungi in the nitrogen nutrition of the host plant. The first section reviews the capacities of these mycorrhizal fungi to influence the N cycle and data regarding the quantification of the N sources in the soil as depending on the ecosystem type. The second section presents the data dealing with the differential capacities of the host-plant and their fungal partners to take up and assimilate the different N sources (organic or mineral). The recent advances gained by molecular studies are detailed. In the third section, labeling experiments and molecular studies that have been carried out so far to highlight N transfer from the fungal cells to the host cells are described. Finally, data enabling us to assess the importance of N transfer in the N budget of the mycorrhizal plants in "real life" are considered.

2 Dynamics of N in Terrestrial Environment and Availability of N in Soils

2.1 The N Cycle from a Mycorrhizal Point of View

In most soils, insoluble organic N constitutes the dominant form of N present (Attiwill and Adams 1993). This N pool is available to plants only after processing into smaller soluble compounds by enzyme cleavage (Jones et al. 2005) that is one of the first steps of the N cycle. These N-containing macromolecules are insoluble and are often protected or coprecipitated with other organic molecules such as lignins, polyphenols and tannins. Thus, these organic molecules have to be degraded by enzymes such as lignases and polyphenol oxidases before that organically-bound N becomes available and can be cleaved by proteases. Lignin can be degraded mainly by "white rot fungi" that are able to produce lignin (LiP) and manganese (MnP) peroxidases. These enzymes digest the aromatic structures of the polymer (Read and Perez-Moreno 2003). The presence of these enzymes has been searched in mycorrhizal fungi that can be cultured in vitro that are ericoid (ERM) and ectomycorrhizal (EM) fungi. Indeed, the capability to produce such enzymes would be of particular relevance in the habitat of these fungi. Ericoid fungi are found in heathland ecosystems characterized by acidic raw humus soils containing high amounts of lignin and polyphenolic compounds. Depending on the climate, EM fungi are also found in boreal forest soils which are characterised by high levels of the same organic molecules. However, so far, studies carried out on the most investigated ERM species, *Hymenosciphus ericae* (Bending and Read 1996, 1997; Burke and Cairney 1998), have failed to find evidence for the production of LiP or MnP. The ability of this fungal species to grow on the aromatic constituents of lignin demonstrated by respirometric methods (Haselwandter et al. 1990) may be attributed to the release of hydrogen peroxide (Bending and Read 1997) and hydroxyl radicals (Burke and Cairney 1998) that can contribute to lignin degradation,

like the "brown rot" fungi (Read and Perez-Moreno 2003). Similarly, the data available on the ability of EM fungi to degrade lignin comes from gene expression studies of MnP (Chambers et al. 1999; Chen et al. 2001) or LiP (Chen et al. 2001) and not from enzyme activity measurements. Contrary to lignin digestion, the extracellular excretion of enzymes such as polyphenol oxidase, peroxidase and laccase that are capable of degrading soil phenolic acids and tannins was clearly demonstrated for ERM and EM fungi (Read and Perez-Moreno 2003; Courty et al. 2005). These observations indicate that those fungal species could play a significant role in organic molecules degradation found in heathland and forest soils. This degradation is a pre-requisite to "unmask" organic N compounds before their cleavage by specialised enzymes, the proteases. In contrast to ERM and ECM fungi, it is considered that the arbuscular mycorrhizal fungi (AMF) have no saprotrophic capabilities to enable N mineralisation from organic matter (Read and Perez-Moreno 2003). So far, there is a single report demonstrating enhanced decomposition of organic necromass by AMF (Hodge et al. 2001). In this experiment, external hyphae of AMF extending from colonized roots were supplied with dual $^{15}N/^{13}C$ labeled grass leaves in a fungal compartment. AM hyphae were shown to facilitate enhancement of N capture from the litter, the N gain in the plants being linearly related to hyphal density in the organic matter. However, this study was not carried out in monoxenic conditions and one cannot exclude that the AM hyphae could act as facilitators, rather than directly, of decomposition and nutrient release. Indeed, AM hyphae are known to increase allocation of carbon from the host-plant to the soil system. This carbon supply could greatly enhance the activity of microbial generalists in the AMF compartment, those microbes that may be involved in decomposition and nutrient release (Read and Perez-Moreno 2003).

The first step of N-containing organic matter digestion is the cleavage of proteins by the proteases. Bending and Read (1996) demonstrated that *H. ericae* was able to acquire N from complex N sources through the expression of extracellular acid protease activities. It is interesting to note that this fungal species is able to grow on a range of N compounds such as chitin, supplied either as crustacean chitin (Leake and Read 1990) or as purified fungal cell wall of *H. ericae* itself (Kerley and Read 1997). Extracellular protease activities have also been measured in ECM fungi (Chalot and Brun 1998; Smith and Read 1997), but at a lower level than those measured in ERM fungi. In addition, availability of N sources and /or climate conditions appears to select ECM fungal species regarding their proteolytic capacities. Northern boreal forests with raw humus soils have ECM fungal communities more diverse and more able to develop on proteins supplied as the sole source of N and C than those isolated from southerly locations where N enrichment occurs (Taylor et al. 2000). Observations made by Tibbett et al. (1998, 1999) on different strains of the ECM fungus *Hebeloma* – greatest proteolytic potential for strains from cold environments and very low thermal optimum for activity of protease (0–6°C) – indicated also that selection may favor "protein fungi" in arctic and boreal environments. After protein hydrolysis, the subsequent step of the N cycle will be the release of NH_4^+ in the environment from amino acids by desaminases. To our knowledge, there is only one study reporting the production of free NH_4^+ into the

medium by an ECM fungus, *Hebeloma crustuliniforme* (Quoreshi et al. 1995). This net release of NH_4^+ occurred only when the fungus was grown without glucose in the medium. The same observation was made on another *Hebeloma* species, *H. cylindrosporum* (Plassard, unpublished data). However, more work is needed to quantify the importance of this ammonium release in situ.

2.2 Quantification of the N Sources in the Soil

N sources that can be used by plants, their fungal symbionts and more generally microorganisms are either mineral (NH_4^+ and NO_3^-) or organic (mainly small peptides and amino acids). Many studies have been undertaken to measure the concentrations of these different N sources in soil. Examples of mean values measured in the soil solution extracted by the drainage/centrifugation method (Christou et al. 2006; Jones et al. 2004, 2006), Rhizon tension lysimeters (Andersson and Berggren 2005) or water incubation (Gessler et al. 1998) are given in Table 1. Although not strictly identical, one can consider that these methods approximate the minimal concentrations of nutrients present in the soil solution. Seasonal variations clearly affect the absolute values of N concentrations (see the Mediterranean vineyard) and boreal climatic conditions clearly decrease N concentration in the solution (Table 1). Finally, under temperate climates, the most abundant N forms are NO_3^- and dissolved organic nitrogen (DON), regarding their concentrations as well as their proportions relative to the total N (Table 1). Surprisingly, NO_3^- concentrations assayed in soil sampled in coniferous species (*Picea sitchensis* and *Larix decidua*) are of the same order of magnitude than those assayed in a Mediterranean vineyard. These data indicate that nitrification should be active in these forest soil conditions. However, as underlined by Jones et al. (2005), such large pools of NO_3^- and DON may occur simply because they contain N forms that are not used easily by plants or microorganisms. If the same argument is held for NH_4^+ and free amino acids whose concentrations and proportions to total N are always very low, these two N pools should be used in priority for uptake. Nevertheless, one have to keep in mind that ammonium and free amino acids can be readily adsorbed onto the solid constituents of soil (Barber 1995; Jones and Hodge 1999; Qualls and Richardson 2003; Vieublé Gonod et al. 2006), thus minimizing their concentrations in soil solution. However, more important than the actual size of the relative N pools in soils is the rate of flux through these pools (Jones et al. 2005). Indeed, using ^{15}N-pool-dilution techniques in short-term experiments, it has been shown that the inorganic N pools are subjected to high turnover due to microbial use (Kaye and Hart 1997; Grenon et al. 2005). This is also the case for free amino acids, with half-life expectancy typically ranging from 1 to 12 h, indicating an extremely fast turnover rate (Jones 1999; Jones and Kielland 2002; Jones et al. 2004).

The other factor that has to be taken into account is the high spatio-temporal variability of mineralization processes between the different soil layers, as illustrated in Table 2. In the forest soils studied, the potential mineralization rates of

Table 1 Dissolved inorganic (NO_3^-, NH_4^+) and organic N concentrations measured in soil solution collected from different ecosystems

Soil type	Ecosystem/ plant species	Climate	Measure-ment period	Soil pH[a]	N (mg N/l)				N proportion (% of total N)				Extr. method[b]	Ref.
					NO_3^-	NH_4^+	DON	Free AA	NO_3^-	NH_4^+	DON	Free AA		
	Vineyard	Mediterra-nean	Winter	7.5	10.4	0.83	31	0.31	25.0	1.5	72.8	0.7	CD	Christou et al. 2006
			Autumn	7.3	150	0.20	80	0.18	65.1	0.09	34.7	0.08		
Distric gleysol	Grassland	Oceanic temperate		6.1	4.2[*]	0[c]	9.5[c]	0.75[d]	29.0	0	65.7	5.3	CD	Jones et al. 2004
Eutric cambisol	Grassland			5.7	3.8[*]	0[c]	6.3[c]	0.22[d]	36.8	0	61.0	2.2	CD	
Haplic podzol	Grassland			4.3	0.2[*]	0[c]	2.6[c]	0.4[d]	6.3	0	81.2	12.5	CD	
Humic cambisol	*Quercus robur* *Acer pseudoplatanus*	Oceanic temperate		4.27	21.4	3.4	22.9	1.01	44.0	7.0	47.0	2.0	CD	Jones and Willett 2006
Leptic podzol	*Picea sitchensis* *Larix decidua*			4.66	130.5	12.8	168.5	6.46	41.0	4.0	53.0	2.0	CD	
	Quercus robur *A. pseudoplatanus*			3.9	49.3	0.6	10.9	0.12	80.9	1.0	17.9	0.2	CD	
Calcic cambisol	*Fraxinus excelsior*			7.01	3.8	0.2	4.5	0.53	42.1	2.2	49.8	5.9	CD	
Acidic podsolic	*Betula pendula*	Continental		4.5	4.3[e]	0.78[e]	-		84.6	12.4			W	Gessler et al. 1998
Para brown earth	*Picea abies*			3.8	8.6[e]	0.74[e]	-		92.1	7.9			W	
F-Layer	*P. abies*	Boreal	Yearly		0.038	0.023	1.50	0.015	2.4	1.5	95.2	0.09	RTL	Andersson and Berggren 2005
H-Layer					0.015	0.025	1.60	0.007	0.9	1.5	97.2	0.4		

DON Dissolved organic nitrogen, *Free AA* free amino acids

[a] pH measured in water

[b] Soil solution extracted by centrifugal drainage (*CD*), soil/water incubation (*W*), rhizon tension lysimeter (*RLT*)

[c,d] Graphically estimated values from Fig. 1 ([c]) and Fig. 2 ([d]) at time zero

[e] Average values calculated from Tab. 1

Table 2 Potential rates of NH_4^+ and NO_3^- production in organic layers (*OL, OF, OH*) and upper mineral soil (*UMS*) sampled in pure *Fagus sylvatica* and mixed *F. sylvatica* and *Carpinus betulus* deciduous temperate forests. Rates of mineral N production were measured after laboratory incubation carried out at 28°C for 28 days (data calculated from Aubert et al. 2005)

	Production rate (μg N g^{-1} soil dw day^{-1})			
	NH_4^+ in		NO_3^- in	
Layer	Pure stand	Mixed stand	Pure stand	Mixed stand
OL	750	200	9	3
OF+OH	1250	400	27	9
UMS	60	30	1.2	0.6
OL/UMS	12.5	6.7	7.5	5.0
OF+OH/UMS	21	13	22	15

NH_4^+ production are much faster in organic layers than in upper mineral soil and the same trend is observed for nitrification (Aubert et al. 2005) (Table 2). Generally speaking, the new N sources produced from hydrolysis of complex N sources are subjected to competition between several processes, that can be physical such as adsorption on solid phase and/or biological such as plant or symbiotic fungal uptake or soil microbial utilisation. Therefore, plants and their fungal symbionts may be supplied with pools of N sources of extreme variable sizes, with a high competition for N capture between the numerous organisms living together and the soil itself.

3 Absorption and Assimilation of Nitrogen by the Partners of the Mycorrhizal Symbiosis

3.1 Organic N Forms: Peptides and Amino Acids

The first forms of N that can be used by plants and their associated fungi are organic N compounds such as small peptides and amino acids. Due to its interest to bypass the N mineralization process, an appreciable research effort has been devoted to the capacities of plants to take up organic N. Pioneering work was made by Read and collaborators (Smith and Read 1997) by studying the growth and N accumulation in young seedlings of Ericaceae (Stribley and Read 1980) and *Betula pendula* (Abuzinadah and Read 1989) supplied with different organic N sources. Their results showed the restricted capabilities of the nonmycorrhizal host plants to use single amino acids as well as oligopeptides (Abuzinadah and Read 1989). The same conclusions were drawn from experiments carried out using L-glutamate supplied to *Pinus pinaster* (Plassard et al. 2000) or various amino acids supplied to *Eucalyptus grandis* and *E. maculata* (Turnbull et al. 1996), although the two Eucalypts were able to use L-glutamine as N source. The same results were obtained when complex substrates such as bovine serum albumine (BSA) or gliadin were supplied to nonmycorrhizal plants of *Vaccinium macrocarpon* (Bajwa et al.

1985) or *Pinus contorta* (Finlay et al. 1992), indicating that, at least the few plant species studied exhibited a rather poor capacity to use such N organic sources when nonmycorrhizal. Clearly, these host-plants were able to utilize organic N and/or complex N sources when associated with mycorrhizal fungi (Smith and Read 1997). Indeed, numerous studies indicated that, in contrast to the host plant, amino acids can be an important source of N for fungal growth as demonstrated for several ectomycorrhizal fungi (Scheromm et al. 1990; Finlay et al. 1992; Quoreshi et al. 1995; Plassard et al. 2000; Wipf et al. 2002; Guidot et al. 2005) and the ericoid fungus, *Hymenoscyphus ericae*, although large intraspecific variation was observed for this species (Cairney et al. 2000; Grelet et al. 2005).

Given the potential importance of organic N use in boreal conditions (Näsholm et al. 1998), this question has been reviewed recently (e.g., Lipson and Näsholm 2001; Näsholm and Persson 2001). According to these reviews, substantial effort has been made in two directions: (1) the study of molecular properties of amino acid transporters, and (2) the measurement of kinetic parameters in various organisms. Although most of the properties of amino acid transport systems have been elucidated from studies of yeast (*Saccharomyces cerevisiae*), numerous studies have confirmed the presence of genes encoding similar transporters in plants (i.e., Fischer et al. 1998). A wide range of transporter genes has been identified so far in *Arabidopsis thaliana* that are expressed in roots of the model plant (Näsholm and Persson 2001). These genes encode low affinity general amino acid transporter, high affinity neutral/acid amino acid transporter, proline transporter, high affinity transporter of basic amino acids, lysine/histine transporter and di- and tripeptide transporter, suggesting that the roots can take up a wide range of organic N sources. Molecular studies have been carried out in two ectomycorrhizal species, *Amanita muscaria* and *Hebeloma cylindrosporum* (Table 3). These studies identified a general amino acid acid transporter (AmAAP1) with high affinity for basic amino acids and somewhat lower affinity for neutral and acidic amino acids in *A. muscaria* (Nehls et al. 1999).

In *H. cylindrosporum*, several transporters have been recently identified: HcGAP1, a general amino acid transporter (Wipf et al. 2002); HcPTR2A and HcPTR2B, two dipeptide transporters (Bendjia et al. 2006); and HcOPT1, an oligopeptide transporter (Müller et al. 2007) (Table 3). The other important set of data deals with the measurement of amino acid uptake rates. As summarized by Lipson and Näsholm (2001), various organisms are able to take up amino acids, in various ecosystems, suggesting that organic N could be a suitable N source for host-plant growth besides mineral N. For example, amino acid absorption was demonstrated for several species of ericoid endomycorrhizal (Sokolovski et al. 2002; Midgley et al. 2004) and ectomycorrhizal fungi (Boukcim and Plassard 2003; Chalot and Brun 1998; Wallenda and Read 1999). Recently, the capacity of uptake and transport of two amino acids (L-Gly and L-Glu) by the external hyphae of *Glomus mossae* associated with wheat was shown (Hawkins et al. 2000). In addition to laboratory experiments, recent studies were carried out in the field to assess the actual use of organic N in situ, either in boreal (Persson et al. 2003), oceanic (Bennett and Prescott 2004) or Mediterranean (Warren 2006) conditions. In boreal conditions,

Table 3 N uptake and N metabolism related genes of mycorrhizal fungi which have been cloned and characterized or whose expression has been determined. Corresponding accession numbers (NCBI, http://www.ncbi.nlm.nih.gov/) are given in parentheses

	Ectomycorrhizas		Endomycorrhizas	
	Ascomycete	Basidiomycete	AMF	Other
N uptake				
NO_3^-	*TbNrt2* (AAX18225)	*HcNrt2* (CAB60009)		
NH_4^+	*TbAMT1* (AAL11032)	*HcAMT1* (AAM21926)	*GintAMT1* (AJ880327)	
		HcAMT2 (AAK82416)		
		HcAMT3 (AAK82417)		
		AmAMT2 (AJ642592)		
Amino acids		*HcGAP1* (AANJ2080)		
		AmAAP1 (CAB38005)		
		HcPTR2A (AAZ32401)		
		HcPTR2B (AAZ32402)		
Peptides		*HcOPT1* [Müller et al 2005[a]]		
N assimilation				
NR (EC 1.6.6.3)	*TbNar1* (AAX18225)	*HcNar1* (CAB60010)		
NiR (EC 1.6.6.4)	*TbNir1* (ABI98041)	*HcNir1* (CAB60008)		
GS (EC 6.3.1.2)	*TbGLN1* (AAP23163)	*HcGLN1* (AAK96111)		
GOGAT (EC 1.4.7.1)	*TbGOGAT* (AAL76245)			
NADPH-GDH (EC 14.1.4)	*TbGDH1* (AAG28788)	*HcGDH1* (AAL06075)		

[a] The accession number is not available, instead the reference is given

mineral or organic N sources (arginine, glycine and peptides) labelled with ^{15}N and ^{13}C were injected into the soil and ^{15}N accumulation was measured in roots of *Deschampsia flexuosa*, *Picea abies* and *Vaccinium myrtillus* after short (6-h) or long-term (65-day) incubation (Persson et al. 2003). Significant differences were found between N sources and plant species only after 65 days of labeling. *P. abies* and *V. myrtillus* accumulated more ^{15}N from glycine and peptides than the grass *D. flexuosa*, although arginine was poorly accumulated in the three species. This may be due to the very low mobility of this strongly cationic amino acid, and its large immobilization in the soil (Persson et al. 2003). Use of organic and mineral N by red cedar (*Thuya plicata*), hemlock (*Tsuga heterophylla*) and salal (*Gaultheria shallon*) under oceanic climate was assessed by measuring ^{15}N accumulation in the whole plant after injection of mineral N and organic N (L-glutamic acid, protein and protein-tannin) up to 20 days after injection (Bennett and Prescott 2004). Contrary to the results of Persson et al. (2003), organic N was accessed to a modest degree compared to mineral N sources by all three species, and the ericacous species Salal did not have a greater capacity to utilize protein and protein-tannin-N. Attached roots of six *Eucalyptus* species incubated simultaneously in situ with

dually labeled ^{13}C- and ^{15}N-glycine, $^{15}NH_4^+$ and $^{15}NO_3^-$ were shown to accumulate glycine at lower rates than ammonium (3.4 ± 0.2 and $6.3 \pm 0.4\,\mu mol\ g^{-1}\ h^{-1}$, respectively) and higher rates than nitrate (0.63 ± 0.07), suggesting that organic N uptake might be of considerable importance in the field (Wallenda and Read 1999). However, it should be noticed that ^{15}N accumulation in the root from organic N supply does not mean that it is a good N source. As underlined by Jones et al. (2005), root cell walls have negative charges that can accumulate positive charged compounds such as ammonium and amino acids by adsorption. Also, it was shown that *Pinus pinaster* plants supplied with ^{15}N-labelled glutamate or $^{15}NH_4^+$ for 24 h accumulated ^{15}N from two N sources at the same rates in their roots, although *Pinus pinaster* plants grew very poorly with L-glutamate without mycorrhizal association (Plassard et al. 2000). This poor growth was due to a very low flux of ^{15}N from L-glutamate towards the shoots, contrary to ammonium. Thus, to assess accurately the actual utilization of organic N by plants, it is necessary to measure accumulation in the roots and in the shoots.

3.2 Mineral N Forms: Ammonium and Nitrate

It is well documented that ectomycorrhizal fungi have a preference for ammonium over nitrate (Rangel-Castro et al. 2002; Guidot et al. 2005), but it is not unusual to find ectomycorrhizal fungi being able to grow better with nitrate over ammonium (Scheromm et al. 1990; Montanini et al. 2002). In contrast, the ectomycorrhizal fungus *Amanita muscaria* is not able to grow with nitrate as the sole source of N. The preferential N source for optimum growth is not only dependent on the species but on the strains studied (Scheromm et al. 1990; Guidot et al. 2005). The same observation was reported for several strains of *Hymenoscyphus ericae* (Cairney et al. 2000; Grelet et al. 2005). We can assume that the strains are adapted to (or have been selected through) the nitrogen availability in the soil from which they have been extracted. These strains must therefore have different ability to take up N.

Early studies conducted on excised and intact ectomycorrhizal root systems evidenced the enhanced ammonium uptake capacity of mycorrhizal plants as compared to their uninfected counterparts (Genetet et al. 1984; Rousseau et al. 1994). An enhanced uptake of nitrate from ectomycorrhizal short roots was also found in the association *Rhizopogon roseolus* and *Pinus pinaster* (Gobert and Plassard 2002), although this positive effect was strongly fungal-species dependent (Plassard et al. 2000). Interestingly, the regulation of nitrate uptake seems to be different in the host-plant and the ectomycorrhizal fungus, as shown in the association *Rhizopogon roseolus* and *Pinus pinaster* (Gobert and Plassard 2002). The fungal nitrate uptake is maximal with or without nitrate in the solution, contrary to the nonmycorrhizal host plant that needs several days of incubation in nitrate solution to reach its maximal level. The kinetics of nitrate uptake into ectomycorrhizal roots as a function of nitrate availability are identical to those established in the fungus alone, suggesting that anion uptake into fungal cells dominate that of root cells in the ectomycorrhizal roots. Such a pattern of uptake could give a broader access to

the ectomycorrhizal plant at fluctuating nitrate availability in the field (Gobert and Plassard 2002).

In addition to the physiological data accumulated on N uptake by the partners and their association, a lot of effort has been devoted to molecular studies in the last decade. The cloning and functional characterization of genes have broadened the knowledge on the pathway of N-uptake and assimilation by mycorrhizal fungi (Table 3). Studies on a few species of ectomycorrhizal fungi start to give a good picture of the molecular actors contributing to the N uptake and assimilation, but very few data are available for the endomycorrhizal fungi and this becomes rarer for the other type of mycorrhizal fungi. *Amanita muscaria (Am)*, *Hebeloma cylindrosporum (Hc)* and *Tuber borchii (Tb)* are the leading ectomycorrhizal fungi concerning the cloning and the expression patterns of the genes coding the proteins involved in inorganic and organic N uptake. Whereas *Hc* and *Am* are phylogenetically close (Basidiomycetes, Agaricales), *Tb* is far apart (Ascomycetes). The strains studied are coming from various types of soils. The *Tuber* strain comes from a calcareous soil poor in organic matter (good mineralization), the *Hebeloma* strain comes from a sandy soil (poor mineralization) and the *Amanita* strain comes from a temperate forest (poor mineralization). *Hc* and *Tb* possess a high affinity NO_3^- transporter Nrt2 (Jargeat et al. 2003; Montanini et al. 2006). Gobert and Plassard (2002) have shown that the ectomycorrhizal fungus *Rhizopogon roseolus* (Basidiomycetes) displays only one kinetics of NO_3^- uptake. The Nrt2 transporter identified so far might therefore be the only high affinity transporter in these fungal species in contrast to the plants that have several transporters. Genes encoding Nitrate Reductase (EC 1.6.6.3) and Nitrite Reductase (EC 1.6.6.4) have also been cloned from *Hc* and *Tb* (Table 3). All three genes are clustered in both fungi. *Tb/HcNrt2* and *Tb/HcNir1* are upregulated in presence of NO_3^- and even under N starvation whereas *TbNr*1 is only upregulated in presence of NO_3^- (Jargeat et al. 2000; Guescini et al. 2007). All three genes are under ammonium repression in *Hc,* but it seems that *TbNr1* is not regulated in the same way. *TbNr1*, *TbNrt2* are strongly expressed in the Hartig net and mantle but weakly expressed into the free-living mycelia (Guescini et al. 2003; Montanini et al. 2006).

Like plants, AMT transporters are responsible for the NH_4^+ uptake in fungi. Three genes encoding transporters have been cloned from *Hc* (Javelle et al. 2001, 2003) and a single one from *Tb* (Montanini et al. 2002), *Am* (Willmann et al. 2007) and *Glomus intraradices* (Lopez-Pedrosa et al. 2006). HcAmt3 is a low affinity ammonium transporter but HcAmt1 and HcAmt2 are high affinity ammonium transporters/sensors. *HcAMT1* is expressed under N deficiency and NO_3^- feeding and repressed by glutamine. *HcAMT3* is highly expressed but not highly regulated. In *Tuber*, *TbAMT1* seems to be only upregulated under N deficiency. The upregulation of *TbAMT1* after transfer of *Tb* in −N solution is slow (matter of days) but its downregulation after N resupplementation is fast (matter of hours). NH_4^+, NO_3^- and glutamine downregulate the transporter (certainly via the glutamine pool as in *Hc*) but proline has no effect (*TbAMT1* remains upregulated) showing that proline is a poor N source as in yeast and *Hc*. In the case of *Amanita*, the gene coding for a high affinity ammonium transporter, *AmAMT2*, is upregulated in −N and nitrate solution

as for *HcAMT1*. The *Amanita* strain studied is not able to use nitrate. *AmAmt1* expression decreases with increasing concentrations of ammonium in the medium with a basal expression reached at 500 µM NH_4^+ as is the case for *TbAMT1*. The expression is further reduced during the formation of ectomycorrhizal roots with the same level of expression in the mantle and the Hartig net. On the contrary, the expression was high in the extraradical mycelium. The last *AMT* member cloned from mycorrhizal fungi is *GintAMT1* from *Glomus intraradices* (Lopez-Pedrosa et al. 2006). It codes for a high affinity ammonium transporter upregulated in low N avaibility and nitrate solution. The downregulation of *GintAMT1* is correlated with increasing concentration of NH_4^+. The transcription of the AMT transporters from mycorrhizal fungi is regulated by the N status of the plant (via the level of glutamine). It is interesting to see that two fungi whose growth is slow on nitrate produce high level of expression of the *AMT* in nitrate solution but the picture is not clear for *Glomus* and *Tuber*. Indeed, these nitrate users seem to possess different regulation. *GintAMT1* expression is regulated as the previous fungi mentioned and *TbAMT1* expression is downregulated under nitrate. However, the *Glomus* study does not include a starvation step contrary to the other ectomycorrhizal fungi.

NH_4^+ produced by nitrate reduction or taken up from the external medium has to be incorporated in C skeleton. This can occur through two ways involving glutamine synthase and glutamine oxoglutarate amino-transferase (GS/GOGAT pathway) or glutamate deshydrogenase and glutamine synthase (GDH/GS pathway). Numerous studies involving these enzymes have been done in different fungi (Bedell et al. 1994; Martin et al. 1994; Javelle et al. 2003; Morel et al. 2006). The GDH/GS pathway seems to dominate in mycorrhizal fungi but the GS/GOGAT dominates in plants. However, depending on the fungal species, GDH activities and polypeptides can be detected or not in ectomycorrhizal roots (Botton and Dell 1994). Interestingly, the fungal aspartate aminotransferase activity was not detected in ectomycorrhizae whereas the activity of root isoenzymes was stimulated (Botton and Dell 1994). Taken all together, these data indicate that expression of enzymes involved in primary assimilation of ammonium in fungal and plant compartments is highly regulated by the mycorrhizal status.

4 Transfer of Nitrogen to the Host Plant

4.1 Direct Methods to Study the Transfer

The demonstration of a net nitrogen transfer from the fungal cells to the root cells was first established by supplying mineral N sources labelled with ^{15}N to the hyphae of an ectomycorrhizal fungus physically separated from the roots of pine seedlings by a barrier (Melin and Nilsson 1952). More recently, Finlay et al. (1988) showed also that the hyphae of four ectomycorrhizal species, *Rhizopogon roseolus,*

Suillus bovinus, Paxillus involutus and *Pisolithus tinctorius,* associated with *Pinus sylvestris* seedlings, were able to take up, assimilate and translocate N from ^{15}N labelled NH_4^+. Labeling was measured in the free amino acid fraction extracted from mycelium (above the barrier), mycorrhizal tips, roots and needles. Whatever the fungal species, the greatest ^{15}N abundancy was measured in the pools of alanine, glutamate + glutamine and aspartate + asparagine, in the mycelium and mycorrhizal tips. The same trend of ^{15}N labelling was found in plant tissues, although the fungal species clearly affected the difference between ^{15}N abundancy measured in fungal and plant compartment (Finlay et al. 1988). The same experimental system was then used by several workers (Finlay et al. 1989; Arnebrandt et al. 1993; Ek et al. 1996; Ek 1997), with different ^{15}N sources and combinations of fungal species and host plant (Table 4). Another experimental system made of two PVC tubes (diameters of 10 and 15 cm) forming a cylindrical inner (fungal) and a ring-shaped outer (plant) compartment was used by Brandes et al. (1998) and Jentschke et al. (2001) to grow the association *Picea abies*/*Paxillus involutus* (Table 4). The inner compartment was accessible only to the hyphae through a nylon mesh. In this system, both compartments were filled with pure sand and nutritive solutions were supplied separately to the plant and the fungus enabling controlled addition of nutrients. Also, the solutions draining from each compartment were collected separately, enabling the measurement of several parameters to follow mineral acquisition by the plant and the fungus. N transfer between plants connected together by the same mycelium was also studied, especially between a N_2-fixing and a non-N_2-fixing tree either using a microcosm (Arnebrandt et al. 1993) or a two-chambered pot made with a nylon mesh (He et al. 2004, 2005). As shown in Table 4, it was possible to measure ^{15}N accumulation in the receiver plant in various experimental conditions. In microcosm studies, the labeling period was rather short (matter of days) and amounts of ^{15}N translocated from NH_4^+ were greater than that translocated from NO_3^- (Ek 1997; Finlay et al. 1989) although the two mineral sources were supplied at the same concentration. Also, more translocated ^{15}N was retained in the roots when nitrate was supplied (Finlay et al. 1989).

When two receiver plants were connected with the same mycelium (Ek et al. 1996), the plant species receiving the largest amount of ^{15}N also supplied most of the carbohydrates to the fungus, suggesting that the regulation of N and C exchanges between the plant and the fungus could be linked together. A more complex situation consisted of studying N exchanges between non-nitrogen- and nitrogen-fixing host plants (Arnebrandt et al. 1993). When ^{15}N-NH_4^+ was supplied to the mycelium of *P. involutus* connected to *Pinus contorta* and non-fixing *Alnus glutinosa*, ^{15}N flux was clearly directed to *Pinus contorta* only. Surprisingly, ^{15}N translocation to *A. glutinosa* was detectable when the host plant was nitrogen fixing (Table 4). When microcosms were exposed to $^{15}N_2$ gas, interplant translocation of ^{15}N was observed, amounting to 7% of the total $^{15}N_2$ fixed (calculation based on Fig. 2 in Arnebrandt et al. 1993). This demonstration of a N transfer between a N_2-fixing plant to a non-fixing plant is, to our knowledge, a unique one. The main limit of the studies carried out in microcosm is the duration of the labeling period that is generally short. This was overcome by using containers with two chambers, separating either the roots

Table 4 N transfer (% of supplied source) and percentage of N derived from transfer (*NFDT*) between ectomycorrhizal fungal–plant or plant–fungal–plant combinations as a function of N source supplied and experimental conditions used

Donor		Receiver (1)	Receiver (2)	Supplied ^{15}N			Total amount of ^{15}N (μmol) in		^{15}N in shoots in shoots (% total ^{15}N)				
Plant	Fungus	Plant		Form	Amount (μmol)	Time	Donor plant	Receiver plant	Donor plant	Receiver plant	N transfer (%)	NDFT (%)	Ref
	Paxillus involutus	*Fagus sylvatica*		$^{15}N\text{-}NH_4^+$	67	3 days		2.8	-	17			Finlay et al. 1989
				$^{15}N\text{-}NO_3^-$	67	3 days		1.8	-	10			
	Scleroderma citrinum	*Betula pendula*		$^{15}N\text{-}NH_4^+$	67	3 days		3.57	-	22			Ek et al. 1996
		Picea abies			67			0.2	-	50			
	P. involutus	*Betula pendula*		$^{15}N\text{-}NH_4^+$	67	4 days		12	-	-			Ek 1997
				$^{15}N\text{-}NO_3^-$	67			5.9	-	-			
	P. involutus	*Pinus contorta*		$^{15}N\text{-}NH_4^+$	67	7 days		2.8	-	50			
			Alnus glutinosa F–[a]					0	-	0			Arneberandt et al. 1993
			Alnus glutinosa F+[a]					0.7	-	25			
A. glutinosa F+	*P. involutus*	*P. contorta*		$^{15}N_2$		7 days	1.85	0.13	58	38	7		
	P. involutus +P[b]	*Picea abies*		$^{15}NH_4^+$	500	1 week		4.9		29		80	Brandes et al. 1998
	P. involutus –P[b]	*Picea abies*		$^{15}N\text{-}NH_4^+$ $^{14}NO_3^-$	77	11 weeks		18.6				1.6	Jetschke et al. 2001
	P. involutus +P	*Picea abies*			77	11 weeks		70				11.5	
Casuarina[c] F–		*Eucalyptus*[d]		$^{15}N\text{-}NH_4^+$	400	4 weeks	69	0.4	-	-	0.6	0.9	He et al. 2004

(continued)

Table 4 (continued)

Donor		Receiver (1)	Receiver (2)	Supplied ^{15}N			Total amount of ^{15}N (µmol) in		^{15}N in shoots in shoots (% total ^{15}N)				
Plant	Fungus	Plant		Form	Amount (µmol)	Time	Donor plant	Receiver plant	Donor plant	Receiver plant	N transfer (%)	NDFT (%)[c]	Ref
Casuarina F–	*Pisolithus* sp.	*Eucalyptus*					93	1.2	-	-	1.3	2.1	
Casuarina F+	*P.* sp.	*Eucalyptus*					114	2.8	-	-	2.4	5.9	
Casuarina F–		*Eucalyptus*		$^{15}N\text{-}NH_4^+$	1333	4 weeks	400	1.14	7.1	31	0.3	1.6	He et al. 2005
Casuarina F–	*P.* sp.	*Eucalyptus*		$^{14}NO_3^-$			391	20.1	8.5	4	3.3	6.0	
Casuarina F+	*P.* sp.	*Eucalyptus*					489	58.6	8	7	10.1	32.1	
Eucalyptus		*Casuarina* F–		$^{15}N\text{-}NH_4^+$	400	4 weeks	118	0.33	-	-	0.3	0.3	He et al. 2004
Eucalyptus	*P.* sp.	*Casuarina* F–					90	1.2	-	-	1.0	1.0	
Eucalyptus	*P.* sp.	*Casuarina* F+					63	8.2	-	-	11.3	7.0	
Eucalyptus		*Casuarina* F–		$^{15}N\text{-}NH_4^+$	1333	4 weeks	136	1.2	42	24	0.9	0.5	He et al. 2005
Eucalyptus	*P.* sp.	*Casuarina* F–		$^{14}NO_3^-$			112	9.1	24	6	10.5	4.3	
Eucalyptus	*P.* sp.	*Casuarina* F+					87	69	14	5	39.1	29.1	
Casuarina F–		*Eucalyptus*		$^{15}N\text{-}NO_3^-$	400	4 weeks	147	0.9	-	-	0.7	1.0	He et al. 2004
Casuarina F–	*P.* sp.	*Eucalyptus*					115	1.7	-	-	1.4	2.1	
Casuarina F+	*P.* sp.	*Eucalyptus*					128	4.5	-	-	3.5	8.6	
Casuarina F–		*Eucalyptus*		$^{15}N\text{-}NO_3^-$	1333	4 weeks	701	7	6	9	1.0	2.7	He et al. 2005
Casuarina F–	*P.* sp.	*Eucalyptus*		$^{14}NH_4^+$			841	17	8	7	2.2	6.3	
Casuarina F+	*P. sp.*	*Eucalyptus*					1037	43	9	9	5.3	28.3	
Eucalyptus		*Casuarina* F–		$^{15}N\text{-}NO_3^-$	400	4 weeks	141	1	-	-	0.7	0.7	He et al. 2004
Eucalyptus	*P.* sp..	*Casuarina* F–					121	2.4	-	-	2.0	1.7	
Eucalyptus	*P.* sp.	*Casuarina* F+					111	16.2	-	-	12.7	7.3	
Eucalyptus		*Casuarina* F–		$^{15}N\text{-}NO_3^-$	1333	4 weeks	180	2.5	36	9	1.3	0.7	He et al. 2005
Eucalyptus	*P.* sp.	*Casuarina* F–		$^{14}NH_4^+$			197	6.7	24	23	5.1	3.6	
Eucalyptus	*P.* sp.	*Casuarina* F+					150	48.6	17	25	23.6	30.2	

[a] *F*– without N_2 fixation, *F*+ with N_2 fixation due to *Frankia* inoculation

[b] +P with Phosphorus supply –P without Phosphorus supply

[c] *Casuarina : C. cunninghamian*

[d] *Eucalyptus: E. maculata*

from the hyphae (Brandes et al. 1998; Jentschke et al. 2001) or the roots from two plant species connected by a common fungus (He et al. 2004, 2005). The main problem is to control exchange of nutrient by mass flow between the compartments through the nylon net. For example, this was done by adding greater solution volume to the plant compartment, creating a supression-promoting mass flow from the plant to the hyphal compartment, and preventing mass flow in the opposite direction. The presence of a negligible mass flow from hyphal to plant compartment was further demonstrated using aluminium as a tracer (Brandes et al. 1998). Using this sophisticated device, Brandes et al. (1998) showed that the addition of NH_4^+ (together with P) to the hyphal compartment for 14 weeks increased the total amount of N in the host-plant by 76%. This figure was confirmed by adding ^{15}N-NH_4^+ during the last week of growth (Table 4), as the percentage of N derived from transfer in the mycorrhizal plants was estimated to be 81%. These results indicate that the mycelium of *P. involutus* was able to take up and to translocate substantial amounts of N from the hyphal compartment to the N-starved host plant that did not have access to the N source. Additional studies were carried out by the same group by supplying a complete nutritive solution containing NH_4NO_3 0.3 mM to the plant (Jentschke et al. 2001). The hyphal compartment was supplied with the same nutritive solution with or without P (Table 4). Irrespective the calculation method (mass balance or ^{15}N labelling), the translocation of N from the fungus to the roots ceased in absence of phosphorus (Table 4). This indicates that the translocation of non-limiting nutrient depends on simultaneous translocation of P (Jentschke et al. 2001). More recently, He and colleagues (Jentschke et al. 2004, 2005) studied N exchanges between a non-fixing (*Eucalyptus maculata*) and a N_2-fixing (*Casuarina cunninghamiana* associated with *Frankia*) species connected together by the same ectomycorrhizal fungus, *Pisolithus tinctorius*. Nitrate and ammonium (4 mM of mineral N) were supplied separately for 5 months (He et al. 2004) or together for 11 months (He et al. 2005), to both plants grown in two-chamber compartmented pot. N transfer between compartments was studied in plants mycorrhizal or not, nodulated or not.

Mycorrhizal symbiosis and nitrogen fixation increased N accumulation by around 2 and 3, respectively, in *Casuarina* plants, whereas mycorrhizal symbiosis increased by around 1.4 and 1.8 N accumulation in *Eucalyptus* plants grown with non-nodulated and nodulated *Casuarina,* respectively (He et al. 2004, 2005). When ^{15}N labelling was applied to nonmycorrhizal donor plants, ^{15}N transfer to the receiver plant was low, irrespective of the N source (Table 4), indicating that the separation efficiently prevented the diffusion of solution between the two compartments. The percentage of N transfer from *Casuarina*, whether fixating or not, to *Eucalyptus* was rather low with values ranging from 1 to 5%, except when plants were fed with ^{15}N NH_4^+-NO_3^- (Table 4). However, due to the differences in the total N amount of the plants (*Casuarina* >> *Eucalyptus*), N derived from transfer from nodulated *Casuarina* amounted to one-third of *Eucalyptus* N when both N sources were supplied together (Table 4). Similarly, the percentage of N transfer from *Eucalyptus* to non-nodulated *Casuarina* was rather low irrespective of the N source (Table 4). However, nodulation of *Casuarina* always dramatically increased N

transfer from *Eucalyptus* (Table 4). Taking into account the bilateral exchanges, the main sink of N taken up by the mycelium was clearly *Casuarina* associated with *Frankia*, whatever the N source (Table 5). Thus, in experimental conditions where *Eucalyptus* and *Casuarina* plants were not starved for N, these data show that the contribution of the net N translocation between plants through the mycelium is very low. Surprisingly, the main sink of translocated N was the plant already containing the greatest amounts of N. As previously shown by Arnebrandt et al. (1993), the direction of these net N exchanges in favour of a N_2-fixing plant could be due to the interdependence of nitrogen and phosphorus shown in the ectomycorrhizal symbiosis (Jentschke et al. 2001). Indeed, the biological nitrogen fixation is a high P-demanding process (Israel 1987). Greater P requirements in nodulated *Casuarina* than in *Eucalyptus* could be filled up by a greater hyphal transport of P and a greater simultaneous translocation of N.

Many studies have also been carried out in plants associated with endomycorrhizal fungi, especially AM species (Smith and Read 1997). The experimental devices used so far are based on containers with one root compartment separated from one or several hyphal compartments by a nylon mesh (e.g. Frey and Schüepp 1992, 1993; Hawkins et al. 1999, 2000; Johansen and Jensen 1996; Johansen et al. 1992, 1993, 1994; Tobar et al. 1994) or an impermeable membrane in polytetrafluoroethylene (PTFE) (Mäder et al. 2000) separating one root compartment from a hyphal one. Whereas these containers have been used to grow whole plants, transport of nitrogen by the hyphae of AM fungi was also studied using Ri T-DNA transformed carrot roots grown in vitro in two-compartment Petri dishes (Hawkins et al. 2000; Bago et al. 2001; Toussaint et al. 2004; Jin et al. 2005). As observed for ectomycorrhizal plants, the main problem is to control the transfer of nitrogen between hyphal and root compartments by mass flow, especially if nitrate is produced by nitrification from $^{15}N\text{-}NH_4^+$ (Johansen et al. 1992) or is supplied as the labeled N source (Tanaka and Yano 2005). This was achieved either by adding N-serve, a nitrification inhibitor (Johansen et al. 1992), using drought conditions (Tobar et al. 1994) or an air gap besides the nylon mesh (Johansen and Jensen 1996; Tanaka and Yano 2005). The use of a PTFE membrane reduced by 86% the passive ^{15}N transport observed with a soil compartment system with nylon mesh alone and an additional root-free buffer zone (Frey et al. 1998). However, a measurable nitrate flux from the hyphal to the root compartment was shown to occur in nonmycorrhizal plants (Mäder et al. 2000). Obviously, the bicompartmented Petri dish is the best device as it makes it possible to control strictly the solution supplied to the external hyphae and the root and to follow its fate throughout the hyphae towards the root. The main limit of this experimental system consists of the absence of shoots. Uptake and translocation of ^{15}N was demonstrated from NH_4^+ and for NO_3^-, supplied separately or together. As shown by Johansen et al. (1994), plant N status greatly influenced the magnitude of N translocation from $^{15}N\text{-}NH_4^+$ between *Glomus intraradices* and *Cucumis sativus* roots. The highest N translocation was observed in plants with the lowest N status, with 49 and 27% of the applied ^{15}N recovered in mycorrhizal plants supplied with 100 and 400 mg N, respectively (Johansen et al. 1994). This greater N translocation was in agreement with the

Table 5 Estimation of the contribution of N transfer between *Eucalyptus maculata* and *Casuarina cunninghamian* connected together via the ectomycorrhizal fungus *Pisolithus* sp. (*Myc*+) or not (*Myc*−). *Casuarina* plants were inoculated (*Nod*+) or not (*Nod*−) with *Frankia*. The donor plant was fed with different inorganic N sources

Status	N source	Sink plant	Net N transfer (mg/plant)[a]	Total N (mg/plant)[b]	N transfer/ Total N (%)	N source	Sink plant	Net N transfer (mg/plant)[c]	Total N (mg/plant)[d]	N transfer/ total N (%)
Myc−, Nod−	$^{15}NH_4^+$	*Eucalyptus*	+ 0.7	144	0.5	$^{15}NH_4^+ - NO_3^-$	*Casuarina*	+ 0.96	375	0.3
Myc+, Nod−		*Eucalyptus*	+ 1.1	166	0.6		*Casuarina*	+ 2.24	750	0.3
Myc+, Nod+		*Casuarina*	+ 26.3	625	4.2		*Casuarina*	+ 125	1,312	9.5
Myc−, Nod−	$^{15}NO_3^-$	Neither	0		0	$^{15}NO_3^- - NH_4^+$	*Eucalyptus*	+ 0.96	187	0.5
Myc+, Nod−		*Casuarina*	+ 0.5	242	0.2		*Eucalyptus*	+ 2.24	312	0.7
Myc+, Nod+		*Casuarina*	+ 26	686	3.8		*Casuarina*	+ 188	1,343	14

[a] Data given in He et al. (2004: Table 5)

[b] Amount of total N in sink plant calculated from the parameters given in He et al. (2004: Table 5), using the equation Total N = [(N transfer (%) × 100)/NDFT(%)]

[c] Values estimated graphically from He et al. (2005: Fig. 5)

[d] Amount of total N in the sink plant calculated using the same equation as in [b] and the parameters N transfer (%) and NDFT(%) given in He et al. (2005: Table 5)

greatest growth improvment of plants due to mycorrhizal colonization. The same trend was observed regarding the magnitude of ^{15}N transfer through *Glomus mossae* hyphae to tomato plants from uniformly labeled NH_4NO_3 (Mäder et al. 2000). Plants were grown at two N fertilizer concentration (NH_4NO_3 at 1 mM (low N) or 3 mM (high N)) in the root compartment. Flux of ^{15}N from the hyphal compartment was estimated to contribute to ca. 42 and 24% of the total N of mycorrhizal plants fed with solution containing a low and a high N fertilizer concentration, respectively. As in ectomycorrhizal plants, potential transfer of N between N_2 fixing and non fixing plants connected by a common AM mycelium was studied. Nodulated *Trifolium alexandrinum* roots connected to maize plants via *Glomus intraradices* hyphae were supplied with $^{15}N_2$ for 5 days (Frey and Schüepp 1992). Although ^{15}N excess measured in maize connected with trifolium via VAM was greater than that measured in nonconnected plants, the amounts of ^{15}N transferred were small, accounting for less than 4% of the ^{15}N fixed by *Trifolium* plants (Frey and Schüepp 1992). Net N transfer of N between *Pisum sativum* and *Hordeum vulgare* associated with *G. intraradices* grown in compartmented devices was also studied when NH_4^+ was supplied to the roots of either species (Johansen and Jensen 1996). Net transfer of ^{15}N from NH_4^+ supplied to pea roots was detected in barley when plants are associated with a mycorrhizal fungus. However, value of N derived from transfer was very low (0.6%). In addition, this net transfer was almost insignificant since N was also transferred in the reverse direction. Net ^{15}N transfer to the receiver plant increased when the shoots of the donor plant were removed, amounting to 15% of the donor-root N after 18 days, while nonmycorrhizal receiver plants contained only 4% (Johansen and Jensen 1996). These data are in agreement with the observation reported by Johanssen et al. (1994) who found that *G. intraradices* hyphae adjacent to heavily labeled roots of cucumber contained very low amounts of ^{15}N, indicating that only small amounts of N were transfered from the roots to the external hyphae. Thus, the net transport of N appears to be much higher from the hyphae to the living roots than in the reverse direction. This could explain the limited role of AM-mycorrhizal hyphae in interplant N transfer.

4.2 N Transfer: Translocated Compounds and Molecular Events

The use of mass spectrometry coupled to the supply of ^{15}N labeled substrates specifically to the fungus made it possible to follow the enrichment of individual amino acid all the way from the extramatrical hyphae to the shoot of the host in ectomycorrhizal (Finlay et al. 1988, 1989; Arnebrandt et al. 1993) or AM (Govindarajulu et al. 2005; Jin et al. 2005) associations. In ectomycorrhizal plants, a feeding period of 73 h with ^{15}N-NH_4^+ resulted in high proportions of ^{15}N–labeled free glutamate/glutamine, aspartate/asparagine, and alanine in the mycelium of *Rhizopogon roseolus*, *Suillus bovinus*, *Pisolithus tinctorius*, and *Paxillus involutus*, although labeled aspartate/asparagine were not found in this last species (Finlay et al. 1988). The same results were found when $^{15}NH_4^+$ or $^{15}NO_3^-$ was supplied to

the hyphae of *P. involutus* associated with *Fagus sylvatica* (Finlay et al. 1989). However, high labeling was also found in aspartate/asparagine in the mycelium (Finlay et al. 1989). Other measurements carried out with *P. involutus* associated with two host plants (*Pinus contorta* and *Alnus glutinosa*) showed that citrulline and glutamine were the amino acids with the highest ^{15}N concentrations in the mycelium and in all parts of the system when $^{15}NH_4^+$ was supplied (Arnebrandt et al. 1993). Thus, these data strongly suggested that the form of N translocation throughout the hyphae could be glutamate/glutamine (Chalot and Brun 1998). The most convincing data about the mechanisms involved in translocation of N in the hyphae and release at the hyphae-host cell interface have been obtained in AM symbiosis (Govindarajulu et al. 2005; Jin et al. 2005). It was first proposed by Bago et al (2001) that the synthesis of arginine and its breakdown through the urea cycle could ensure an efficient net translocation of N together with the recycling of carbon. This novel metabolic pathway was nicely demonstrated by using the association AM-carrot roots grown in vitro in two-compartment Petri dishes. The labeling with stable isotopes showed that arginine, representing more than 90% of the total free amino acid pool in the extraradical mycelium (ERM) became heavily labeled after the supply of $^{15}NO_3^-$ or $^{15}NH_4^+$. In addition to ^{15}N supply, the ERM was fed with $^{13}C_2$ acetate. Free amino acids in the ERM became heavily labeled, with 34–77% of the molecules of the three abundant amino acids (arginine, glutamate and aspartate) having one or more ^{13}C atoms. In contrast, proteins from the colonized roots did not become detectably labeled with ^{13}C under these conditions, strongly suggesting that no carbon is transferred to the host cell. Feeding the ERM with $^{13}C_{U6}$ arginine indicated that arginine was transported intact from the ERM towards the IRM and showed the absence of ^{13}C arginine labeling in the proteins extracted from the roots. In contract, supplying ^{15}N arginine to the ERM resulted in ^{15}N labeling in all the free amino acids in the root tissue, including those present at high levels in uncolonized roots and at low levels in the ERM. Taken together, these data confirmed that (1) the nitrogen but not the carbon of arginine is transferred from fungus to host accross the host-fungus interface (Govindarajulu et al. 2005; Jin et al. 2005), and (2) arginine must be broken down to release NH_4^+. This could be ensured by the operation of arginase, releasing ornithine and urea, and urease releasing NH_4^+ and CO_2 from urea. Ornithine (and NH_4^+) could be then metabolized through ornithine amino transferase (OAT) for amino acid synthesis in the IRM. This metabolic route is supported by a higher expression of genes putatively encoding fungal urease, OAT and ammonium transporter in IRM (in mycorrhizal roots) than in ERM (Govindarajulu et al. 2005).

From the available data in the literature, it is clear that the mechanisms of exchange of N between fungal and plant cells are not yet fully understood. Exchanges of N mean that N-containing molecules or ions have to permeate through the fungal plasma membrane because there is no direct connection between the cytosol of fungal and plant cells, such as plasmodesmata making the cortical symplasm. So far, our knowledge about the solutes (identification and concentration) present in the apoplastic space are completely lacking and very difficult to obtain experimentally. The use of molecular approaches, offering a tool to study specifically the expression

of genes from fungal or plant origin in the mycorrhizal tissue, appears as an invaluable strategy to gain new insights about the pathways involved in both partners and thus hypothesize which N compounds are transferred at the fungus–root interface. As stated before, ammonium resulting from the breakdown of translocated arginine is transferred at the root–fungus interface in AM symbiosis. However, as discussed by Chalot et al. (2006), the fungal *Amt* gene which was found overexpressed in IRM compared to ERM is not compatible with a release of NH_4^+ into the apoplast through the encoded Amt transporter. Rather, such a transporter would ensure the reuptake of any molecule of ammonium from the apoplast back to the fungal cytosol, resulting in a futile cycling of inorganic N and probably in an inefficient N transfer (Chalot et al. 2006). In addition, in ectomycorrhizal symbiosis, even if a net translocation of ^{15}N in the shoots of the host-plant was demonstrated, the pathway of N translocation and exchange probably does not involve arginine because, contrary to AM hyphae, this amino acid is not a major form of N accumulation in the mycelium or in the mycorrhizal root (see, e.g., Finley et al. 1989). Therefore, glutamine and/or asparagine appear as the best candidates to sustain the metabolic pathway leading to N release into the apoplastic space of Hartig net. Nevertheless, it should be noticed that urea was assayed in ectomycorrhizal tips (ECM) and in extraradical mycelium (EM) of *Paxillus involutus* associated with *Betula pendula* seedlings grown in microscosms (Morel et al. 2005). The concentration of urea was the same in ECM and EM (1 µmol/g dwt) and constituted the second main N compound in ECM (the first one was glutamate) and the main N compound in EM. These data suggest that urea cycle could also be involved in N transfer. However, nontargeted studies did not confirm this hypothesis. Messenger RNAs extracted from ECM and EM were hybridized to microarrays made from genes specifically expressed in the free-living mycelium fed with Gln (N derepression, i.e., low internal amino acid content) or with NH_4^+ (N repression, i.e., high internal amino acid content). The results obtained showed that 66 genes (of 1,200 used to make the microarray) were found to be differentially expressed in ECM and EM. In ECM, the gene that was the most upregulated (24-fold) was identified as encoding a putative phosphatidylserine decarboxylase (Psd), an enzyme which could contribute to membrane remodeling during ectomycorrhiza formation. Surprisingly, genes coding for urea (Dur 3) and spermine (Tpo3) transporters were upregulated 4.1- and 6.2-fold in EM. Morel et al. (2005) hypothesize that these transporters could facilitate the translocation of nitrogen compounds within the EM network. It is possible that the selection of a given fungal gene population realized in the fungus grown in pure culture hampered the discovery of the genes involved in N exchanges. However, it should be noticed that genes with no known homology were also found to be upregulated in ECM and in EM. These genes of unknown function might be those involved in N exchanges, underlying the need to discover their function.

The molecular data available so far indicate that organic N (amino acids) as well as inorganic N (NH_3/NH_4^+) could be exchanged in ectomycorrhizal symbiosis (Table 6). Acting in favor of organic N exchange is the strong repression of *HcGAP1* (coding a broad amino acid spectrum transporter) in ectomycorrhizal roots of *Hebeloma cylindrosporum–Pinus pinaster*. The release of amino acid could be ensured by a

Table 6 Candidate N molecules and genes which could be involved in N exchanges between fungal and plant cells at the Hartig net level in ectomycorrhizal roots

	Fungal side				Plant side			
Transfered N molecules	Gene	Function	Status	Ref	Gene	Function	Status	Ref
Amino acids	*HcGAP1*	Amino acid transporter in *Hebeloma cylindrosporum*	Fully repressed in ECM	Wipf et al. 2002	*PpAAP1* to *4*	Amino acid permease from *Pinus pinaster*	Not yet characterized	Olivi et al., unpublished, in Müller et al. 2007
	ScAqr1	Excretion of aa in yeast	Not yet cloned in mycorrhizal fungi	Müller et al. 2007				
NH_4^+ / NH_3	Ato3	Outward amonium transporter in yeast	Homologs cloned in *Amanita muscaria* (AJ644726, AJ644793)	U. Nehls, unpublished, in Selle et al. 2005	*PttAMT1.2*	High affinity Ammonium importer from *Populus tremula* × *tremuloides*	Characterized in yeast Overexpressed in ECM	Selle et al. 2005
					PoptrAMT1.2b *PoptrAMT1.4a* *PoptrAMT1.3*	Ammonium importer from *Populus trichocarpa*	Not yet characterized Overexpressed in ECM	Selle et al. 2005

transporter from the same family as Aqr1 recently characterized in *Saccharomyces cerevisiae*, which is involved in excretion of amino acids (Chalot et al. 2006; Müller et al. 2007). Its homolog could be searched in EST libraries (*Hebeloma cylindrosporum*; Wipf et al. 2003; Lambilliotte et al. 2004) and in the database coming from sequencing project of the ectomycorrhizal fungus *Laccaria bicolor* (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). In addition, amino acid permeases of the host-plant, *P. pinaster*, have been identified. Their functional characterization and their pattern of expression in ECM will greatly help to know whether or not the corresponding transporters could play a role to take up the released organic N in the apoplastic space. On the other hand, ammonium release at the level of Hartig net is also supported by results obtained with the association between *Amanita muscaria* and Poplar roots (Table 6; Selle et al. 2005). As indicated by these authors, two genes homologous to a supposed ammonium export protein of *S. cerevisiae*, Ato3, are expressed in *A. muscaria* (Table 6). In agreement with the putative efflux of NH_4^+ into the apoplast, the *Populus* high affinity ammonium transporter gene *PttAMT1.2* was found overexpressed in ectomycorrhizal roots. Three other *Populus* genes coding putative ammonium transporters were also found to be overexpressed in ECM (Selle et al. 2005).

However, the form of inorganic N (NH_3 or NH_4^+) and the molecular mechanism responsible for inorganic N efflux are still matter of debate. Chalot et al. (2006) proposed several pathways that could be followed by inorganic N to be extruded from fungal cells, either by passive efflux of the deprotonated form or by protein-mediated mechanisms. Alternatively, excess ammonia could be stored in intracellular vesicles, either by diffusion of NH_3 or by active transport of NH_4^+ (Chalot et al. 2006). It is important to keep in mind that, due to the pKa of NH_3 (9.25), the value of cytosolic pH will have a huge effect on the ratio NH_4^+:NH_3. For example, [NH_4^+] will be 177- or 44-fold higher than [NH_3] in the cytosol at pH 7 and 7.5, respectively. It is generally assumed that cytosolic pH is around 7, indicating that NH_4^+ must be largely predominant over NH_3. On the other hand, due to its potential decoupling activity, free NH_4^+ cannot accumulate to a great extent in the cytosol. Thus, the active transport of NH_4^+ via an Amt-mediated transport system into acidified vesicles would ensure the compartmentalization of excess of inorganic N, as proposed by Chalot et al. (2006). These NH_4^+-loaded vesicles could then move via microtubules to the symbiotic membrane where they would fuse with the plasma membrane and release ammonium into the interfacial apoplast (Chalot et al. 2006). Voltage-dependent cation channels such as proposed for export of fixed NH_4^+ between endosymbiotic rhizobia bacteria and the legume host (Roberts and Tyerman 2002) could be involved in export of inorganic N from the symbiotic cell (Chalot et al. 2006).

4.3 N Transfer in Mycorrhizal Plants: The Situation in the Real Life

Despite the fact that the delivery of nitrogen taken up by the fungus to the plant, whether associated with ecto- or AM-mycorrhizal fungi, was demonstrated many times, data in the literature also questioned the quantitative contribution of hyphal

N transfer on whole N plant budget. In the ectomycorrhizal symbiosis, for example, the data published with the association between *Hebeloma cylindrosporum* and *Pinus pinaster* illustrate this point. Grown in containers with a high N concentration (5 mM) supplied either as NH_4^+, NO_3^- or L-glutamate, nonmycorrhizal plants grew better than mycorrhizal ones, except with organic N supply where the reverse situation was observed. Plants fed with mineral N accumulated less N than nonmycorrhizal ones. Measurement of biomass and N accumulation in the fungus showed that considerable amounts of N were immobilized in the fungus, whatever the N source (Plassard et al. 2000). Therefore, these results questioned the occurrence of N transfer between the fungus and the host, particularly when nitrate was supplied. However, recent molecular studies quantified the level of transcripts from plant and fungal nitrite reductases, and fungal ammonium transporter HcAmt1 in control uninoculated roots, extraradical mycelia and ECM formed by either wild type or nitrate reductase deficient fungal strains (Bailly et al. 2007). Supplied with 1 mM NO_3^-, the plant nitrite reductase was found repressed by the two wild type strains and fully expressed by the nitrate reductase-deficient strain. As the plant nitrite reductase is highly repressed by reduced N and fully expressed by NO_3^-, these data strongly suggest that this fungal species is able to translocate reduced N to the host plant after NO_3^- reduction by fungal nitrate and nitrite reductases. In contrast, the positive effect of ectomycorrhizal symbiosis on growth and N accumulation by the host plant has been clearly demonstrated in early experiments carried out with organic N as the N source (see Section 2.1). However, in this case, an indirect effect of the fungus on N utilization by the plant cannot be excluded. Fungal cells could release NH_4^+ into the medium from the hydrolysis of L-glutamate (as shown in pure culture; see Quoreshi et al. 1995). Available NH_4^+ could then be taken up directly by the roots grown in the same medium, explaining the better growth of mycorrhizal plants. In addition, it is clear that variations among fungal species might exist regarding their efficiency to translocate N to their host plant. However, as shown in Table 4, most of the studies aiming at quantifying N transfer between the fungus and the host plant have been only carried out on a few species such as *Paxillus involutus* or *Pisolithus tinctorius*. Although not specifically designed to assess the N transfer between the mycelium and the host plant, a recent study carried out in the field by Nara (2005) indicates strong differences between species to help young seedlings of *Salix reinii* connected to mother plants to grow in volcanic desert at Mount Fuji, Japan (Nara 2005). Eleven native fungal species were used to connect 1-year mother plant with young seedlings of *S. reinii*. After 5 months of growth, young seedlings were harvested and their biomass, N and P contents measured. Results obtained showed that nutrient acquisition and growth of seedlings connected to mycelial network were improved with most fungal species. From these data, we did the following calculations: firstly, we divided the total amount of N and P accumulated in the whole seedlings by the root dry weight to get the accumulation rates of N and P by taking into account the effect of mycorrhizal symbiosis on root growth; secondly, we divided each N and P accumulation rate by that of nonmycorrhizal control plants. Such a ratio will give indication on the possible supplementary N or P accumulation in the host plant through mycelial uptake and/or translocation of nutrients from the mother tree. As shown in Fig. 1, the effect of

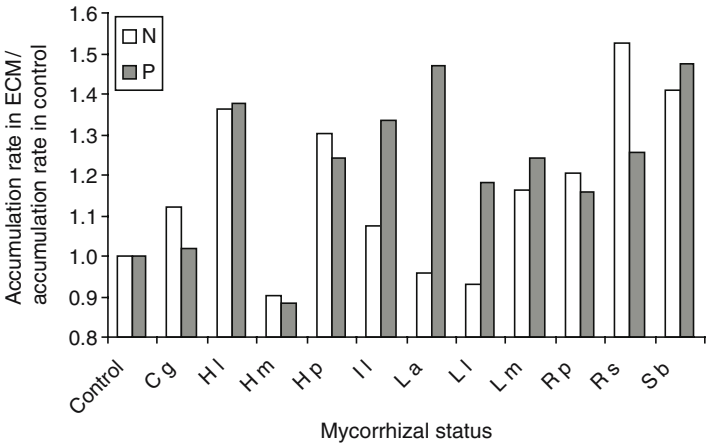

Fig. 1 Variability of enhancement of N and P accumulation rates in young seedlings of *Salix reinii* connected to mother trees via different native ectomycorrhizal species using data published by Nara (2005). Given are the ratios calculated between accumulation rates of N or P (total amounts of N or P in whole seedlings divided by root dry weigth) in ectomycorrhizal plants to that of nonmycorrhizal (control) plant. A ratio <1 indicates a negative effect whereas a ratio >1 indicates a positive effect of ectomycorrhizal symbiosis. The ectomycorrhizal species were *Cg Cenococcum geophilum, Hl Hebeloma leucosarx, Hm H. mesophaeum, Hp H. pusillum, Il Inocybe lacera, La Laccaria amethystina, Ll L. laccata, Lm Laccaria murina, Rp Russula pectinatoides, R.s R. sororia, Sb Scleroderma bovista*

individual species varied strongly among them, with: a ratio <1 only for N (*Laccaria amethystina, L. laccata*), suggesting a net transfer of P but not of N; a ratio <1 for N and P (*Hebeloma mesophaeum*), suggesting no net transfer of nutrients for this species; a ratio >1 for N and P (*Hebeloma leucosarx, H. pusillum, Laccaria murina, Russula pectinatoides, R. Sororia, Scleroderma bovista*), indicating simultaneous transfer of N and P; or a ratio slightly >1 for N (*Cenococcum geophilum* and *Inocybe lacera*) suggesting a low effect on N accumulation that is independent of a P effect. These data clearly indicate that interspecific variations of efficiency in N (and P) capture and translocation exist among the fungal species in situ and raise the question of the extrapolation of results from a few "laboratory" species (such as *Paxillus involutus* or *Pisolithus* sp.) to the real world.

The same question can be raised for AM plants. Indeed, studies carried out with in vitro system showed clearly that huge amounts of N taken up by extraradical hyphae are transferred to the carrot roots (up to 50%; Govindarajulu et al. 2005). However, a set of published data indicates that the contribution of AM to the N nutrition of the host plant is low. For example, the study of Reynolds et al. (2005) manipulated N availability (control vs inorganic and organic forms) and AM species (control vs four AM species) for five old-field perennials grown individually in a glasshouse under N-limiting conditions. Although not quantifying N transfer between the fungus and the plant, the results showed that AM fungi were at best neutral and that some AM species depressed growth for some plant species, suggesting

that AM fungi did not promote N acquisition at low N supply. However, the fact that this study was carried out in a constrained rooting volume may have favored the competition between the hyphae and the root for N uptake, such conditions promoting growth-depressing effects of AM fungi in responsive plant species. In another study, Hawkins et al. (1999) showed that additional supply of NH_4NO_3 (2 mM) only to the hyphae of *Glomus mossae* increased by 14% the total amount of N in wheat plants, with 84.1 and 73.5 mg N/plant in mycorrhizal and nonmycorrhizal plants, respectively, giving a net increase of 10.6 mg N/plant due to AM. On the other hand, AM-plants supplied with NH_4NO_3 2 mM in both the hyphal and root compartment contained 50% more N than NM plants with 371 and 250 mg N/plant in AM and control plants, respectively. From their results, the authors concluded that mycorrhizal N uptake may help plants to survive during periods of low soil N supply but is not a mechanism to sustain high plant growth rates in agricultural or horticultural crops. However, it should be underlined that AM plants make a better use of N supplied at a high concentration than nonmycorrhizal ones, contrary to what was observed in ectomycorrhizal *Pinus pinaster* plants (see above). The same type of experiment was carried out with NO_3^- as the sole N source supplied at 2 mM to the hyphal compartment and at 0.2 or 2 mM to the wheat root compartment (Hawkins et al. 2000). In this case, there was no significant difference between the shoot or root dry weight or N concentration of AM- and non-AM-plants (Hawkins et al. 2000). These results are in agreement with those published by Tanaka and Yano (2005) who showed that only 2.9% of the shoot-N of maize plants colonized by *Glomus aggregatum* was derived from nitrate-N added to the hyphal compartment while 74% was derived from the slow-release urea. Curiously, intraradical hyphae isolated from roots contained appreciable amount of ^{15}N in the cell walls even when $^{15}NO_3^-$ was the N source. The main cell wall N compound is chitin that is synthesized from reduced N, indicating that this fungal species is able to assimilate NO_3^-. However, these results are not in agreement with those of Govindarajulu et al. (2005) who showed that ^{15}N from nitrate was translocated from the extraradical hyphae of *Glomus intraradices* to carrot roots. These discrepancies could be explained by a much lower rate of ^{15}N assimilation from ^{15}N-nitrate than from ^{15}N-urea, leading to concentration of amino acids that could be used for ^{15}N translocation such as arginine (Govindarajulu et al. 2005; Jin et al. 2005) much lower than that produced from urea assimilation. In agreement with this hypothesis, intraradical hyphae grown with urea contained 6 times more ^{15}N in their cell walls than those grown with nitrate (Tanaka and Yano 2005). This observation is in favor of a much greater N assimilation from supplied urea than from nitrate.

5 Conclusions

From the available data in the literature, it can be concluded that the mycorrhizal fungi are able to use a variety of N sources as their host-plants, although individual differences exist among fungal and plant capacities. Clear experimental evidence

exists indicating that N taken up by the hyphae in the external medium is translocated throughout the hyphae towards the mycorrhizal root, leaves the fungal cells and is finally taken up by the root cell. However, despite the demonstration of this pathway in AM carrot roots grown in vitro, many questions remained to be answered, especially regarding the molecular mechanisms involved in the exchange of N-containing molecules at the level of root–fungus interface as well as how these mechanisms are regulated. To address these questions, which deal with the identification of molecular events resulting in the exchange of N, two main approaches could be used that can be divided in untargeted versus targeted ones. The untargeted approach, microarray, should take its full power with the complete sequencing and annotation of genomes (i.e., the complete genome of *Laccaria bicolor*, http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html; *Glomus intraradices*, http://www.jgi.doe.gov/sequencing/DOEmicrobes.html; *Populus trichocarpa* (Tuskan et al. 2006). Analysis of the whole genomes of both partners should help us to discover new genes and to study their expression in function of different N supplies. However, the main "bottle-neck" of this approach is the need to establish the function of unknown genes whose expression is specifically modified by the symbiosis. This could be achieved using functional complementation of yeast. Also, the localization of gene expression (IRM, ERM) and localization of the protein in the cell will be helpful in understanding their putative role in the symbiosis. In situ hybridization (Kaldorf et al. 1998), in situ RT-PCR (Van Aarle et al. 2007) or protein labeling using antibodies (Montanini et al. 2006) could be used to gain new information about the localization of any interesting genes. Besides these untargeted approaches, targeted ones could also be used, especially in combination with labeling experiments and differential tissue analysis such as the ones carried out in AM symbiosis (Govindarajulu et al. 2005; Jin et al. 2005). It seems very important to establish the pathways of N transfer as a function of the N source and their concentrations, as those used in laboratory experiments are generally much higher than those assayed in the soil solution (compare Tables 1 and 4). Indeed, if we are able to determine the key metabolites whose concentration determines the efficiency of mycorrhizal symbiosis on N transfer to the host cell, it will be easier to extend our knowledge to other fungal species than the few ones which have been studied over the last years, hence contributing to a better understanding of the ecological role of mycorrhizal symbiosis in the field.

References

Abuzinadah RA, Read DJ (1989) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants IV. The utilization of peptides by birch (*Betula pendula* L.) infected with different mycorrhizal fungi. New Phytol 112:55–60

Andersson P, Berggren D (2005) Amino acids, total organic and inorganic nitrogen in forest floor soil solution at low and high nitrogen input. Water Air Soil Pollut 162:369–384

Arnebrandt K, Ek H, Finlay R, Söderström B (1993) Nitrogen translocation between *Alnus glutinosa* (L.) Gaertn. Seedlings inoculated with *Frankia* sp. and *Pinus contorta* Doug. Ex Loud seedlings connected by a common ectomycorrhizal mycelium. New Phytol 124:231–242

Attiwill PM, Adams MA (1993) Nutrient cycling in forests. New Phytol 124:561–582
Aubert M, Bureau F, Vinceslas-Akpa M (2005) Sources of spatial and temporal variability of inorganic nitrogen in pure and mixed deciduous temperate forests. Soil Biol Biochem 37:67–79
Bago B, Shachar-Hill Y, Pfeffer PE (2001) Could the urea cycle be translocating nitrogen in the arbuscular mycorrhizal symbiosis? New Phytol 149:4–8
Bailly J, Debaud JC, Verner MC, Plassard C, Chalot M, Marmeisse R, Fraissinet-Tachet L (2007) How does a symbiotic fungus modulate expression of its host-plant nitrite reductase? New Phytol 175:155–165.
Bajwa R, Abuarghub S, Read DJ (1985) The biology of mycorrhiza in the Ericaceae. X. The utilization of proteins and the production of proteolytic-enzymes by the mycorrhizal endophyte and by mycorrhizal plants. New Phytol 101:469–486
Barber SA (1995) Soil nutrient availability, a mechanistic approach, 2nd edn. Wiley, New York
Bedell JP, Garnier A, Pireaux JC, Chalot M, Brun A, Botton B (1994) Study of enzymes involved in nitrogen-metabolism of Douglas-*Laccaria laccata* ectomycorrhizas. Acta Bot Gallica 141:483–490
Bending GD, Read DJ (1996) Nitrogen mobilization from protein-polyphenol complex by ericoid mycorrhizal fungi. Soil Biol Biochem 28:1602–1612
Bending GD, Read DJ (1997) Lignin and soluble-phenolic degradation by ectomycorrhizal and ericoid mycorrhizal fungi. Mycol Res 101:1348–1354
Bendjia M, Rikirsch E, Müller T, Morel M, Corratgé C, Zimmermann S, Chalot M, Frommer WB, Wipf D (2006) Peptide uptake in the ectomycorrhizal fungus *Hebeloma cylindrosporum*: characterization of two di- and tri-peptide transporters (HcPTR2A and B). New Phytol 170:401–410
Bennett JN, Prescott CE (2004) Organic and ionrganic nitrogen nutrition of western red cedar, western hemlock and sala in minearl N-limited cedar-hemlock forests. Oecologia 141:468–476
Botton B, Dell B (1994) Expression of glutamate dehydrogenase and aspartate aminotransferase in eucalypt ectomycorrhizas. New Phytol 126:249–257
Boukcim H, Plassard C (2003) Juvenile nitrogen uptake capacities and root architecture of two open-pollinated families of *Picea abies*. Effects of nitrogen source and ectomycorrhizal symbiosis. J Plant Physiol 160:1211–1218
Brandes B, Godbold D, Kuhn A, Jentschke G (1998) Nitrogen and phosphorus acquisition by the mycelium of the ectomycorrhizal fungus *Paxillus involutus* and its effect on host nutrition. New Phytol 140:735–743
Burke RM, Cairney JWG (1998) Carbohydrate oxidases in ericoid and ectomycorrhizal fungi: a possible source of Fenton radicals during degradation of lignocellulose. New Phytol 139:636–645
Cairney JWG, Sawyer NA, Sharples JM, Meharg AA (2000) Intraspecific variation in nitrogen use utilisation by isolates of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan. Soil Biol Biochem 32:1319–1322
Chalot M, Blaudez D, Brun A (2006) Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. Trends Plant Sci 11:263–266
Chalot M, Brun A (1998) Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. FEMS Microb Rev 22:21–44
Chambers SM, Burke RM, Brooka PR, Cairney JWG (1999) Molecular and biochemical evidence for manganese-dependent peroxidase activity in *Tylospora fibrillose*. Mycol Res 103:1098–1102
Chen DM, Taylor AFS, Burke RM, Cairney JWG (2001) Identification of genes for lignin peroxidases and manganese peroxidases in ectomycorrhizal fungi. New Phytol 152:151–158
Christou M, Avramides EJ, Jones DL (2006) Dissolved organic nitrogen dynamics in a mediterranean vineyard soil. Soil Biol Biochem 38:2265–2277
Courty PE, Pritsch K, Schlotter M, Hartmann A, Garbaye J (2005) Activity profiling of ectomycorrhiza communities in two forest soils using multiple enymatic tests. New Phytol 167:309–319
Ek H (1997) The influence of nitrogen fertilization on the carbon economy of *Paxillus involutus* in ectomycorrhizal association with *Betula pendula*. New Phytol 135:133–142

Ek H, Andersson S, Söderström B (1996) Carbon and nitrogen flow in silver birch and Norway spruce connected by a common mycorrhizal mycelium. Mycorrhiza 6:475–467

Finlay RD, Ek H, Odham G, Söderström B (1988) Mycelial uptake, translocation and assimilation of nitrogen from ^{15}N-labelled ammonium by *Pinus sylvestris* infected with four different ectomycorrhizal fungi. New Phytol 110:59–66

Finlay RD, Ek H, Odham G, Söderström B (1989) Uptake, translocation and assimilation of nitrogen from ^{15}N-labelled ammonium and nitrate sources by intact ectomycorrhizal systems of *Fagus sylvatica* infected with *Paxillus involutus*. New Phytol 113:47–55

Finlay RD, Frostegard A, Sonnerfeldt AM (1992) Utilization of organic and inorganic nitrogen-sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl Ex Loud. New Phytol 120:105–115

Fischer W-F, André B, Rentsch D, Krolkiezer S, Tegeder M, Britkreuz K, Frommer WB (1998) Amino acid transport in plants. Trends Plant Sci 3:188–195

Frey B, Schüepp (1992) Transfer of symbiotically fixed nitrogen from berseem (*Trifolium alexandrinum* L.) to maize via vesicular-arbuscular mycorrhizal hyphae. New Phytol 122:447–454

Frey B, Schüepp (1993) Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. New Phytol 124:221–230

Frey B, Brunner I, Christie P, Wiemken A, Mäder P (1998) The use of polytetrafluoroethylene (PTFE) hydrophobic membrane to study transport of ^{15}N by mycorrhizal hyphae. In: Varma A (ed) Mycorrhiza manual. Springer, Heidelberg, pp 151–158

Genetet I, Martin F, Stewart GR (1984) Nitrogen assimilation in mycorrhizas - ammonium assimilation in the N-starved ectomycorrhizal fungus *Cenococcum graniforme*. Plant Physiol 76:395–399

Gessler A, Schneider S, Von Sengbucsh D, Webber P, Hanemann U, Huber C, Rothe A, Kreutzer K, Rennenberg H (1998) Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytol 138:275–285

Gobert A, Plassard C (2002) Differential NO_3^- dependent patterns of NO_3^- uptake in *Pinus pinaster, Rhizopogon roseolus* and their ectomycorrhizal association. New Phytol 154:509–516

Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–823

Grelet GA, Meharg AA, Alexander IJ (2005) Carbon availability affects nitrogen source utilisation by *Hymenoscyphus ericae*. Mycol Res 109:469–477

Grenon F, Bradley RL, Jones M, Shipley B, Peat H (2005) Soil factors controlling mineral N uptake by *Picea engelmanii* seedlings: the importance of gross NH_4^+ production rates. New Phytol 165:791–800

Guescini M, Pierleoni R, Palma F, Zeppa S, Vallorani L, Potenza L, Sacconi C, Giomaro G, Stocchi V (2003) Characterization of the *Tuber borchii* nitrate reductase gene and its role in ectomycorrhizae. Mol Genet Genomics 269:807–816

Guescini M, Zeppa S, Pierleoni R, Sisti D, Stocchi L, Stocchi V (2007) The expression profile of the *Tuber borchii* nitrite reductase suggests its positive contribution to host plant nitrogen nutrition. Curr Genet 51:31–41

Guidot A, Verner MC, Debaud JC, Marmeisse R (2005) Intraspecific variation in use of different organic nitrogen sources by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mycorrhiza 15:167–177

Haselwandter K, Bobleter O, Read DJ (1990) Degradation of 14C-labelled lignin and dehydropolymer of coniferyl alcohol by ericoid and ectomycorrhizal fungi. Arch Microb 153:352–354

Hawkins H-J, Johansen, George E (1999) Effect of plant nitrogen status on the contribution of arbuscular mycorrhizal hyphae to plant nitrogen uptake. Physiol Plant 105:694–700

Hawkins H-J, Johansen, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285

He X, Critchley C, Ng H, Bledsoe C (2004) Reciprocal N ($^{15}NH_4^+$ or $^{15}NO_3^-$) transfer between non-N_2-fixing *Eucalyptus maculata* and N_2-fixing *Casuarina cunninghamiana* linked by the ectomycorrhizal fungus *Pisolithus* sp. New Phytol 163:629–640

He X, Critchley C, Ng H, Bledsoe C (2005) Nodulated N_2-fixing *Casuarina cunninghamiana* is the sink for net N transfer from non-N_2-fixing *Eucalyptus maculata* via an ectomycorrhizal fungus *Pisolithus* sp. Using $^{15}NH_4^+$ or $^{15}NO_3^-$ supplied as ammonium nitrate. New Phytol 176:897–712

Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413:297–299

Israel DW (1987) Investigation of the role of phosphorus in symbiotic dinitrogen fixation. Plant Physiol 84:835–840

Jargeat P, Gay G, Debaud JC, Marmeisse R (2000) Transcription of a nitrate reductase gene isolated from the symbiotic basidiomycete fungus *Hebeloma cylindrosporum* does not require induction by nitrate. Mol Gen Genet 263:948–956

Jargeat P, Rekangalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L (2003) Characterisation and expresion analysis of a nitrate transporter and nitrite reductase genes, two members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Curr Genet 43:199–205

Javelle A, Morel M, Rodriguez-Pastrana BR, Botton B, Andre B, Marini AM, Brun A, Chalot M (2003) Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Mol Microbiol 47:411–430

Javelle A, Rodriguez-Pastrana BR, Jacob C, Botton B, Brun A, Andre B, Marini AM, Chalot M (2001) Molecular characterization of two ammonium transporters from the ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEBS Lett 505:393–398

Jentschke G, Brandes B, Kuhn A, Schröder WH, Godbold D (2001) Interdependance of phosphorus, nitrogen, potassium and magnesium translocation by the ectomycorrhizal fungus *Paxillus involutus*. New Phytol 149:327–337

Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y (2005). The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol 168:687–696

Johansen A, Jakobsen I, Jensen ES (1992) Hyphal transport of ^{15}N-labelled nitrogen by a vesicular-arbsucular mycorrhizal fungus and its effect on depletion of inorganic soil N. New Phytol 122:281–288

Johansen A, Jensen ES (1996) Transfer of N and P from intact or decomposing roots of pea to barley interconnected by an arbuscular mycorrhizal fungus. Soil Biol Biochem 28:73–81

Johansen A, Jakobsen I, Jensen ES (1993) External hyphal of vesicular-arbsucular mycorrhizal fungi associated with *Trifolium subterraneum* L. 3. Hyphal transport of ^{32}P and ^{15}N. New Phytol 124:61–68

Johansen A, Jakobsen I, Jensen ES (1994) Hyphal N transport by a vesicular-arbsucular mycorrhizal fungus associated with cucumber grown at three nitrogen levels. Plant Soil 160:1–9

Jones DL (1999) Amino acid biodegradation and its potential effects on organic nitrogen capture by plants. Soil Biol Biochem 31:613–622

Jones DL, Hodge A (1999) Biodegradation kinetics and sorption reactions of of three differently charged amino acids in soil and their effects on plant organic nitrogen availability. Soil Biol Biochem 31:1331–1342

Jones DL, Kielland K (2002) Soil amino acid turnover dominates the nitrogen flux in permafrost-dominated taiga forest soils. Soil Biol Biochem 34:209–219

Jones DL, Willett VB (2006). Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. Soil Biol Biochem 38:991–999

Jones DL, Shannon D, Murphy DL, Farrar J (2004) Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. Soil Biol Biochem 36:749–756

Jones DL, Healey JR, Willett VB, Farrar JF, Hodge A (2005) Dissolved organic nitrogen uptake by plants-an important N uptake pathway? Soil Biol Biochem 37:413–423

Kaldorf M, Schmelzer E, Bothe H (1998) Expression of maize and fungal nitrate reductase genes in arbuscular mycorrhiza. Mol Plant Microbe Interact 11:439–448

Kaye JP, Hart SC (1997) Competition for nitrogen between plants and soil microorganisms. Trends Ecol Evol 12:139–143

Kerley SJ, Read DJ (1997) The biology of mycorrhiza in the Ericaceae XIX. Fungal mycelium as a nitrogen source for the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kernan and its host plants. New Phytol 136:691–701

Lambilliotte R, Cooke R, Samson D, Fizames C, Gaymard F, Plassard C, Tatry MV, Berger C, Laudie M, Legeai F, Karsenty E, Delseny M, Zimmermann S, Sentenac H (2004) Large-scale identification of genes in the fungus *Hebeloma cylindrosporum* paves the way to molecular analyses of ectomycorrhizal symbiosis. New Phytol 164:505–513

Leake JR, Read DJ (1990) Chitin as a nitrogen source for mycorrhizal fungi. Mycol Res 94:993–1008

Lipson D, Näsholm T (2001) The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. Oecologia 128:305–316

Lopez-Pedrosa A, Gonzalez-Guerrero M, Valderas A, Azcon-Aguilar C, Ferrol N (2006) GintAMT1 encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*. Fungal Genet Biol 43:102–110

Mäder P, Vierheilig, Streitwolf-Engel R, Boller T, Frey B, Christie P, Wiemken A (2000) Transport of ^{15}N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi. New Phytol 146:155–161

Martin F, Cote R, Canet D (1994) NH_4^+ assimilation in the ectomycorrhizal basidiomycete *Laccaria bicolor* (Maire) Orton, a ^{15}N-NMR study. New Phytol 128:479–485

Melin E, Nilsson H (1952) Transport of labelled nitrogen from an ammonium source to pine seedlings through mycorrhizal mycelium. Svensk Bot Tidskr 46:281–285

Midgley DJ, Chambers SM, Cairney JWG (2004) Inorganic and organic substrates as sources of nitrogen and phosphorus for multiple genotypes of two ericoid mycorrhizal fungal taxa from *Woollsia pungens* and *Leucopogon parviflorus* (Ericaceae). Aust J Bot 52:63–71

Montanini B, Moretto N, Soragni E, Percudani R, Ottonello S (2002) A high-affinity ammonium transporter from the mycorrhizal ascomycete *Tuber borchii*. Fungal Genet Biol 36:22–34

Montanini B, Viscomi AR, Bolchi A, Martin Y, Siverio JM, Balestrini R, Bonfante P, Ottonello S (2006) Functional properties and differential mode of regulation of the nitrate transporter from a plant symbiotic ascomycete. Biochem J 394:125–134

Morel M, Jacob C, Kohler A, Johansson T, Martin F, Chalot M, Brun A (2005) Identification of genes differentially expressed in extraradical mycelium and ectomycorrhizal roots during *Paxillus involutus-Betula pendula* ectomycorrhizal symbiosis. Appl Environ Microbiol 71:382–391

Morel M, Buee M, Chalot M, Brun A (2006) NADP-dependent glutamate dehydrogenase: a dispensable function in ectomycorrhizal fungi. New Phytol 169:179–190

Müller T, Avolio M, Olivi M, Benjdia M, Rikirsch E, Kasaras A, Fitz M, Chalot M, Wipf D (2007) Nitrogen transport in the ectomycorrhiza association: The *Hebeloma cylindrosporum-Pinus pinaster* model. Phytochemistry 68:41–51

Nara K (2005) Ectomycorrhizal networks and seedling establishment during early primary succession. New Näsholm T, Persson J (2001) Plant acquisition of organic nitrogen in boreal forests. Physiol Plant 111:419–426

Nehls U, Kleber R, Wiese J, Hampp R (1999) Isolation and characterization of a general amino acid permease Phytol 169:169–178

Näsholm T, Ekblad A, Nordin A, Giesler R, Högberg M, Högberg P (1998) Boreal forest plants take up organic nitrogen. Nature 392:914–916

Nehls U, Kleber R, Wiese J, Hampp R (1999) Isolation and characterization of a general amino acid permease from the ectomycorrhizal fungus: *Amanita muscaria*. New Phytol 144: 343–349

Persson J, Högberg P, Ekblad A, Högberg MN, Nordgren A, Näsholm T (2003) Nitrogen acquisition from inorganic and organic sources by boreal forest plants in the field. Oecologia 137:252–257

Plassard C, Bonafos B, Touraine B (2000) Differential effects of mineral and organic N sources, and of ectomycorrhizal infection by *Hebeloma cylindrosporum*, on growth and N utilization in *Pinus pinaster*. Plant Cell Environ 23:1195–1205

Qualls RG, Richardson CJ (2003) Factors controlling concentration, export, and decomposition of dissolved organic nutrients in the Everglades. Biogeochemistry 62:197–229

Quoreshi AM, Ahmad I, Malloch D, Hellebust J (1995) Nitrogen metabolism in the ectomycorrhizal fungus *Hebeloma crutuliniforme*. New Phytol 131:263–271

Rangel-Castro JI, Danell E, Taylor AF (2002) Use of different nitrogen sources by the edible ectomycorrhizal mushroom *Cantharellus cibarius*. Mycorrhiza 12:131–137

Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol 157:475–492

Reynolds HL, Hartley AE, Vogelsang KM, Bever JD, Schultz PA (2005) Arbuscular mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials under low nitrogen supply in glasshouse culture. New Phytol 167:869–880

Roberts DM, Tyerman SD (2002) Voltage-dependent cation channels permeable to NH_4^+, K^+, and Ca^{2+} in the symbiosome membrane of the model legume *Lotus japonicus*. Plant Physiol 128:370–378

Rousseau JVD, Sylvia DM, Fox AJ (1994) Contribution of ectomycorrhiza to the potential nutrient-absorbing surface of pine. New Phytol 128:639–644

Scheromm P, Plassard C, Salsac L (1990) Effect of nitrate and ammonium nutrition on the metabolism of the ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum* Romagn. New Phytol 114:227–234

Selle A, Willmann M, Grunze N, Gessler A, Weiss M, Nehls U (2005) The high-affinity poplar ammonium importer PttAMT1.2 and its role in ectomycorrhizal symbiosis. New Phytol 168:697–706

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, San Diego

Sokolovski SG, Meharg AA, Maathuis FJM (2002) *Calluna vulgaris* root cells show increased capacity for amino acid uptake when colonized with the mycorrhizal fungus *Hymenoscyphus ericae*. New Phytol 155:525–530

Stribley DP, Read DJ (1980) The biology of mycorrhiza in the Ericaceae VII. The relationship between mycorrhizal infection and the capacity to utilize simple and complex organic nitrogen sources. New Phytol 86:365–371

Tanaka Y, Yano, K (2005) Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. Plant Cell Environ 28:1247–1254

Taylor AFS, Martin F, Read DJ (2000) Fungal diversity in ectomycorrhizal communities of Norway spruce (*Picea abies* (L) Karst) and Beech (*Fagus sylvatica* L) in forests along north-south transects in Europe. In: Schulze E-D (ed) Carbon nitrogen cycling in European forest ecosystems. Ecological studies, vol 142. Springer, Heidelberg, pp 343–365

Tibbett M, Sanders FE, Minto SJ, Dowell M, Cairney JWG (1998) Utilization of organic nitrogen by ectomycorrhizal fungi (*Hebeloma* spp.) of arctic and temperate regions. Mycol Res 102:1525–1532

Tibbett M, Sanders FE, Cairney JWG, Leake JR (1999) Temperature regulation of extracellular proteases in ectomycorrhizal fungi (*Hebeloma* spp.) grown in axenic culture. Mycol Res 103:707–714

Tobar R, Azcon R, Barea JM (1994) Improved nitrogen uptake and transport from ^{15}N-labelled nitrate by external hyphae of arbuscular mycorrhiza under water-stressed conditions. New Phytol 126:119–122

Toussaint JP, St-Arnaut M, Charest C (2004) Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an *in vitro* comparmented system. Can J Microbiol 50:251–260

Turnbull MH, schmidt H, Erskine S, Richards PD, Stewart GR (1996) Root adaptation and nitrogen source acquisition in natural ecosystems. Tree Physiol 16:941–948

Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313:1596–1604

Van Aarle IM, Viennois G, Amenc LK, Tatry MV, Luu DT, Plassard C (2007) Fluorescent *in situ* RT-PCR to visualise the expression of a phosphate transporter gene from an ectomycorrhizal fungus. Mycorrhiza 17:487–494

Vieublé Gonod L, Jones DL, Chenu C (2006) Sorption regulates the fate of the amino acids lysine and leucine in soil aggregates. Eur J Soil Sci 57:320–329

Wallenda T, Read DJ (1999) Kinetics of amino acid uptake by ectomycorrhizal roots. Plant Cell Environ 22:179–187

Warren CR (2006) Potential organic and inorganic N uptake by six *Eucalyptus* species. Funct Plant Biol 33:653–660

Willmann A, Weiss M, Nehls U (2007) Ectomycorrhiza-mediated repression of the high-affinity ammonium importer gene AmAMT2 in *Amanita muscaria*. Curr Genet 51:71–78

Wipf D, Bendjia M, Tegeder M, Frommer WB (2002) Characterization of a general amino acid permease from *Hebeloma cylindrosporum*. FEBS Lett 528:119–124

Wipf D, Bendjia M, Rikirsch E, Zimmermann S, Tegeder M, Frommer WB (2003) An expression cDNA library for suppression cloning in yeast mutants, complementation of a yeast *his4* mutant, and EST analysis from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Genome 46:177–181

Ion Dynamics During the Polarized Growth of Arbuscular Mycorrhizal Fungi: From Presymbiosis to Symbiosis

Alessandro C. Ramos, Arnoldo R. Façanha, and José A. Feijó(✉)

1 Introduction

The 450-million-year-old symbiosis between the majority of land plants and arbuscular mycorrhizal (AM) fungi is one of the most ancient, abundant, and ecologically important symbioses on earth (Remy et al. 1994; Taylor et al. 1995). The obligate biotrophic nature of the AM fungus makes it difficult to study and analyze the fungus in vitro (absence of roots), but it is conceivable that the early events in the evolution of mycorrhizal symbioses may have involved reciprocal genetic changes in ancestral plants and free-living fungi. The prospect makes this system a remarkably interesting one to characterize and fully understand.

The development of AM interaction starts before the physical contact between the host plant roots and the AM fungus. Hyphal growth and branching are induced by the root factors exudated by host plants, and are followed by the formation of an appressorium leading to the hyphal penetration in the root system. These root signaling factors seems to be specifically synthesized by host plants, as exudates from nonhost plants are not able to promote either hyphal differentiation or appressorium formation (Giovannetti et al. 1993, 1996). Most root exudates contain several active compounds including strigolactones that stimulate the hyphal growth and branching (Akiyama et al. 2005; Akiyama and Hayashi 2006; Besserer et al. 2006), but many are still to be characterized (Harrison 2005). Inside the root cortex, the intercellular growing hyphae develop highly branched structures, named arbuscules, which penetrate the root cell wall and then invaginate on the plasma membrane of cortical cells of the host root. At the same time, a specific genetic program is triggered to induce the expression of specific membrane proteins. The resulting modified plant plasma membrane is denominated periarbuscular membrane (PAM). During this step of the symbiotic process, the formation of a modified plant plasma membrane also occurs (Gianinazzi-Pearson 1996; Smith and Read, 1997; Harrison 2005; Ramos et al. 2005).

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Fast and directed hyphal growth of AM fungal germ tubes is required in order to reach the host root and begin the colonization. Hyphae are tip-growing cells that sense subtle root-originating signals and environmental changes, and respond by changing their growth axis, a signaling feature shared with other tip-growing organisms (Feijó et al. 2004). Likewise, the structural organization of germ tubes of AM fungi is typical of tip-growing cell elongation, namely in the polarized cytological organization of the nucleus and organelles, streaming, etc.

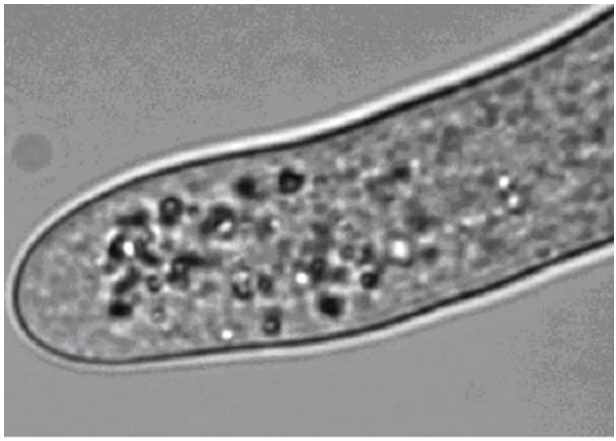
Evidence that the fungal growth and host signal perception are mediated by ion fluxes across the plasma membrane have recently accumulated, and in most cases the participation of proton (H^+) fluxes seem to be consistently involved. During plant and fungal growth, cells extrude H^+ can out of their cells through two different mechanisms: (1) the activity of P-type plasma membrane H^+-ATPases (Palmgren 2001), and (2) plasma membrane redox reactions (Barr 1991; Kim et al. 2007). The exact proportions in which both mechanisms contribute is not exactly known, but in most plant cells the plasma membrane H^+-ATPase seems to be essential in terms of most of the vital processes in a cell. Fungal cells also energize their plasma membrane using P-type H^+-pumps quite similar to the plant ones. Some genes of P-type H^+-ATPase have been isolated from symbiotic fungi (Ferrol et al. 2000; Requena et al. 2003), but only two (GmHA5 and GmPMA1) have proved to encode H^+-ATPases expressed in *Glomus mosseae* (Corradi et al. 2004). Previously, an ATP hydrolysis activity was described in AM fungi and cytochemical analysis for this activity localized it mainly in the first 70 μm from the tip (Lei et al. 1991). This is in line with data obtained with a H^+-specific vibrating probe, which indicated that the H^+ efflux in *Gigaspora margarita* is more concentrated in the subapical region of the hyphae (Ramos et al., 2008). Moreover, having in mind the correlation between the cytosolic pH profile (Jolicoeur et al. 1998) with the vanadate-sensitive H^+ efflux pattern, it seems clear that a H^+-ATPase enzyme is activated in the plasma membrane at the subapical region during the presymbiotic stages of AM fungi development. The rationale for this kind of signaling assumption that H^+, very much like Ca^{2+} ions, may act as second messengers, where extra- and intracellular transient pH changes are preconditions for certain processes (e.g., the gravity response or activation of certain transporters in stomatal movements). Taken together, the available evidence could be enough to suggest the hypothesis that extracellular H^+ fluxes could constitute a "pH signaling" signature that would promote the electrical phenomena related to the host recognition, appressorium formation and root colonization. This review aims at defining the boundaries of such a hypothesis. We offer a comprehensive review of the polarized growth of AM fungal hyphae, but from the particular perspective of putative regulatory processes that may influence or be influenced by extracellular H^+-fluxes, cytosolic pH and membrane potential. These in turn will be contextualized in terms of cytoplasmic dynamics and other effects of signaling and regulatory compounds. A role for H^+ dynamics as an emergent signaling pathway will be discussed as an important mechanism in the ionic dialogue established between AM fungi and higher plants.

2 The Structural Organization Definition and Polarized Growth in the Germ Tubes of AM Fungi

Tip-growing cells display a typically polarized cell growth. This phenomenon is based on the occurrence of cell elongation strictly at the apex, which is maintained by newly synthesized membrane delivered by vectorial exocytosis. There are several different examples of cells that exhibit polarized growth, including pollen tubes, root hairs, algal rhizoids, and fungal hyphae of filamentous fungi (Hepler et al. 2001). Some of these are evidently the fastest growing cells in nature (Feijó et al. 2004). Generally, cells extend continuously at their extreme tips, where enzymes and raw wall materials are released into the extracellular microenvironment. In the case of branching hyphae, the stabilization of the nascent polarity axis may be the critical step that permits the emergence of a new hypha (Harris 2006). It is supported by the continuous movement of materials into the tip mobilized from older regions. This polarized growth is the hallmark of filamentous fungi (Harris and Momany 2004) conferring a "penetrating power", and may constitute the main reason by which they can recognize signals and colonize plants.

The ultrastructure, intracellular dynamic of organelles and the study of storage and secretion progress in germ tubes of AM fungi have already been examined in the presymbiosic stage (Sward 1981a, 1981b; Siqueira et al. 1985; Astrom et al. 1994; Maia and Kimbrough 1994; Maia et al. 1994; Bago et al. 1998, 1999b), but remains a puzzle if compared with other organisms where it has been studied in detail, such as *Neurospora crassa* (Seiler and Plamann 2003; Harris and Momany 2004; Harris 2006), pollen tubes (Feijó et al. 1995; Hepler et al. 2001; Cheung and Wu 2007) and root hairs (Campanoni and Blatt 2007).

A schematic diagram of a germ tube of *Gigaspora margarita* (Fig. 1) illustrates the typical cytoplasmic organization essential for polarized growth in these hyphae. The germ tube of *Gigaspora margarita* is similar to other organisms with tip-growing cells, i.e., characterized by three regions designated as apical, subapical and vacuolated (more distal zone), also characteristic of some filamentous fungi (Seiler and Plamann 2003). One difference observed is the presence of various small vacuoles and not just a single large one as occurs in mature plant cells. The apical region (first 5 µm from the tip) is characterized by a single-layered primary wall, lacking organelles, but containing numerous vesicles. The presence of the secondary wall starts in the subapical region, which contains several organelles including a large nucleus, mitochondria, endoplasmic reticulum, vacuoles, microtubules and a diversity of dark vesicles or dense granules. The last region of vacuolation contains some organelles present in the subapical region, but is remarkable in the high number of vacuoles that increase in size with the tip distance (see details in Sward 1981b). Furthermore, germ tubes of *Gigaspora. margarita* labeled with Oregon Green 488 showed the presence of two kinds of vacuoles, including spherical and tubular shapes (<0.5 µm diameter). Tubular vacuoles are the most conspicuous subcellular structure longitudinally oriented along the germ tube and appear to be closely associated with cytoplasmic streaming (Uetake et al. 2002). Bi-directional



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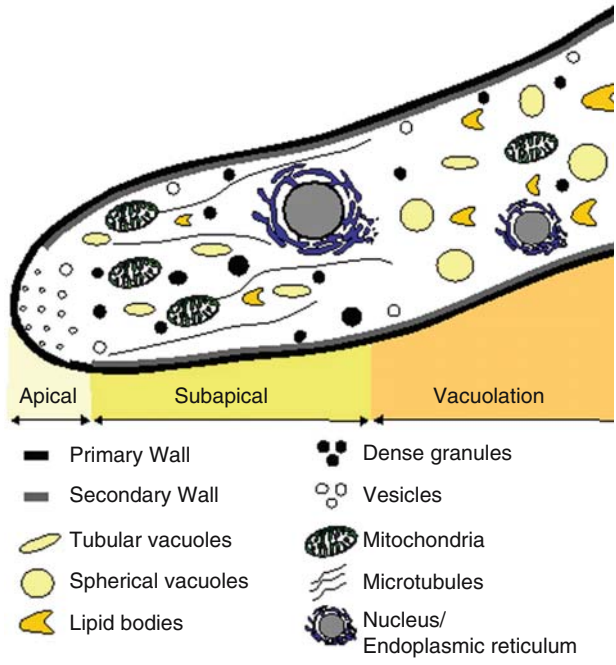

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Fig. 1 **a** Light micrograph of a *Gigaspora margarita* germ tube showing dense granules, some vesicles and a clear zone at the tip. **b** Diagrammatical representation of the ultrastructural organization of *G. margarita* defining its apical growth

movement of nuclei (Bago et al. 1998, 1999b) and acid vesicles has also been found in the cytoplasm of growing hyphae (Saito et al. 2004).

Time-lapse video microscopy of the germ tube growth reveals that a cyclic movement of organelles (vacuoles, vesicles and dense granules) is concentrated

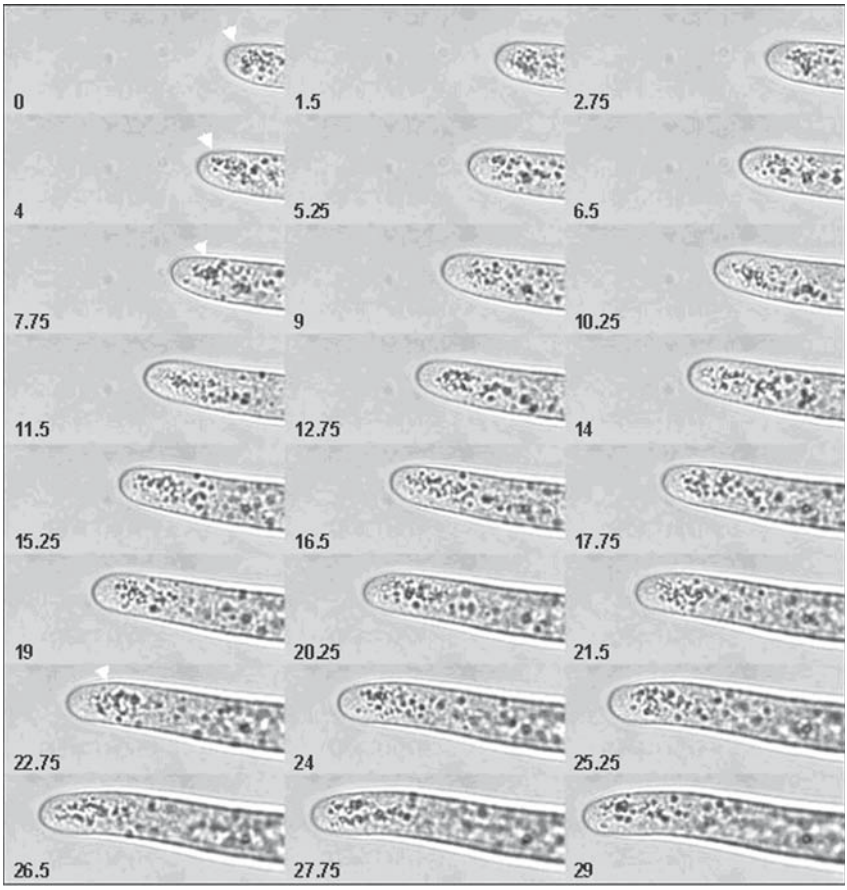

Fig. 2 Time-lapse video microscopy of the hyphal growth of *Gigaspora margarita* during the presymbiosis state. The pictures show intense cytoplasmic streaming and movement of clear vesicles and dense granules ("Spitzenkörper-like" structures) observed mainly at the hyphal subapical region (*arrowheads*). The *numbers* represent the recorded time expressed in minutes

mainly at the subapical region, 5–40 μm from the tip (Fig. 2). Generally speaking, filamentous fungi contain specific apical bodies called "Spitzenkörpers", consisting of a cluster of small membrane-bound vesicles embedded in a meshwork of actin microfilaments. In pathogenic fungi, Spitzenkörpers are strictly located in the hyphae apical region and there is evidence that they play a role in steering the growth process (Bartnicki-Garcia et al. 2000; Riquelme and Bartnicki-Garcia 2004; Harris et al. 2005). The vesicle supply centre (VSC) model of polarized growth in filamentous fungi proposes that the Spitzenkörper is the repository for secretary vesicles that are transported along hyphae towards the tip. Vesicles radiate from the Spitzenkörper and travel to the cell surface, where they fuse with the plasma membrane and release their cargo (Crampin et al. 2005). Electron-microscopy analysis revealed Spitzenkörper-like structures in AM fungi that were defined only as dark

vesicles or dense granules by Sward (1981b). In agreement with this, Astrom et al. (1994) used indirect immunofluorescence microscopy in *Glomus mosseae* hyphae to detect microtubules in both central and cortical regions of the hyphae, while actin microfilaments were resolved as actin-like structures. They found plaques on the microfilaments of actin and suggested that these plaques could be small vesicles associated with actin as described in the Spitzenkörpers of other fungi (Astrom et al. 1994; Bartnicki-Garcia et al. 2000). However, it was not possible to establish clearly the position of the actin microfilaments inside the germ tube, which makes it uncertain if these actin-associated vesicles are localized either in apical or subapical regions of AM fungi. The subapical region has been considered the specialized hyphal elongation zone, where most of the apparatus and organelles involved in growth process are concentrated, namely mitochondria, lipid bodies and glycogen migrated from the anterior parts of the tube (Bago et al. 1999a, 2000; Lammers et al. 2001; Bago et al. 2002a,b).

3 Proton Fluxes and the Regulation of Ion Uptake

Tip-growing plant cells, as AM hyphae, can also exhibit a marked polarization on internal gradients of free cytolosic concentration of, and/or external fluxes of, important regulatory ions (e.g., calcium, protons and chloride), which appear to play a fundamental role in the establishment and/or maintenance of the polarized growth habit (Boavida et al. 2005).

Certain ions have been known for a long time to be important second messengers in important signaling pathways (Feijó et al. 1999; Holdaway-Clarke and Hepler 2003; Hepler 2005). Calcium (Ca^{2+}) has received particular attention, mostly because of the so-called "Ca^{2+} signature" typical in many physiological processes (in plants, see, e.g., Cárdenas et al. 1999; Hetherington and Brownlee 2004; Hepler 2005). This term refers to the transient spatial and temporal changes which characterizes a specific cell-signaling event. On the other hand, life as we know it occurs in aqueous media. As water spontaneously ionizes, cells live in a "proton world" and any change in the pH of the cell environment provokes an impact on a variety of molecules in different ways. H^+ are likely to be important contributors to plant cell-associated electrical currents (Miller and Gow 1989; Miller et al. 1991; Felle 2001), and its flux is important to cytosolic pH regulation, secondary active transporter of organic and inorganic nutrients, turgor-regulation, and in the regulation of cell wall plasticity, as suggested in "Acid-Growth Theory" (Rayle and Cleland 1992; Cleland 1993). Proton gradients and their intimate association with a polarized growth were described in pollen tubes (Feijó et al. 1999, 2001; Holdaway-Clarke and Hepler 2003), and there is increasing evidence that protons may also be functionally important as regulatory signaling or effector molecules (Felle 2001; Vissenberg et al. 2001; Felle et al. 2004). Moreover, protons in coordination with other ions like chloride seem to have specific roles in the process of cell growth (Zonia et al. 2002).

New technologies and methodologies for the study of ion transport in higher plants and fungi cells advanced the understanding of the ion dynamic mechanisms by which these organisms uptake and translocate ions (Miller and Gow 1989). Cell nutrition and growth in fungi and plants are dependent on proton electrochemical gradient generated mainly by the plasma membrane H^+-ATPases (Portillo 2000; Palmgren 2001; Sondergaard et al. 2004). This enzyme belongs to a multigene family of P-type H^+ pumps that couples the hydrolysis of ATP to the H^+ transport. They were initially described as exclusive to the plasma membrane of plants and fungi cells (Palmgren 2001), but more recently they were also found in some flagellate protists (Scott and Docampo 1998). The H^+ electrochemical gradient generated provides a driving force for the uptake of various nutrient and solutes (Palmgren 2001), but it has been proposed that it can also act as an intermediate in certain signal transduction pathways (Xing et al. 1996). In summary, the P-type H^+-ATPase is an electrogenic enzyme and it extrudes H^+ that accumulate on the outside of the cell, thereby decreasing the apoplast pH, and comprises the primary system of proton transport of all plant and fungal cells. This primary energization of the membrane is then used for a number of other cotransporters that use some sort of chemo-osmotic feature to carry other molecules spontaneously through a variety of channel proteins. In fact, most of the hundreds of membrane-bound transport proteins that have been identified in plants and fungi are thought to be energized indirectly through the action of P-type H^+-ATPases (Palmgren 2001).

Recently we have used a proton-selective vibrating probe to analyze the H^+ dynamics in spores and germ tubes of *G. margarita*. In living spores of AM fungi, most cells show a polarized profile where H^+ influxes are localized in the region of the spore opposed to the site of germ tube emergence, while H^+ effluxes are found in the region where the germ tubes emerge (Fig. 3). These fluxes are correlated with the direction and magnitude of electrical currents described previously for each germination stage of AM spores (Berbara et al. 1995). In addition, the electrical membrane potential (Em) across the spore wall from non-germinated spores was found to be more negative than in germinated spores (Ayling et al. 2000). On the other hand, Chloride (Cl^-) displays the inverse direction of H^+, going out in regions where H^+ goes in, and vice versa (Fig. 3). This suggests that regulation of germ tubes emergence is under the control of polarized distribution of H^+ fluxes, and the direction of Cl^- fluxes could explain the hydration phenomenon, where water is preferentially entering into the spore.

The extracellular H^+ flux around elongating hyphae reaches the maximal intensity of efflux at distance of 10–40 µm (subapical region) and then decreases to basal levels at the tip and over the next 200 µm (Fig. 4). Some functional evidence suggests a characteristic distribution of the proton pumps activity predominantly around the subapical region of the hyphal tube, but apparently absent in their apex. A pharmacological study shows that those H^+ effluxes are susceptible to the addition of specifics inhibitor of the P-type H^+-ATPase (sodium orthovanadate), which abolished the hyphal H^+ effluxes, mainly in the sub-apical region, but exerted no effect in the apical region (Fig. 4). Jolicoeur et al. (1998), using BCECF-AM dye (a ratiometric dye) measurements, reported that the cytosolic pH profile of

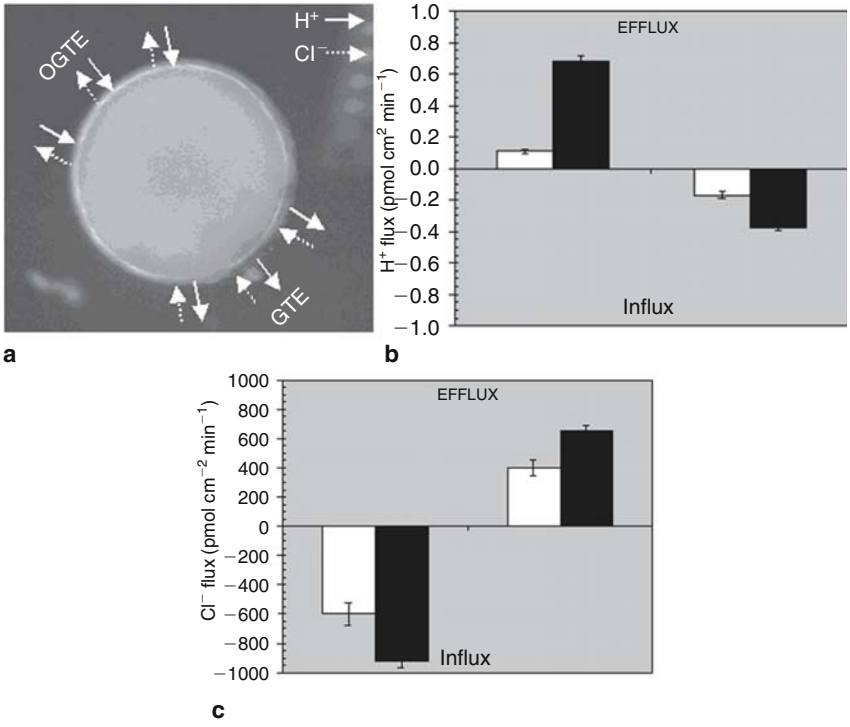

Fig. 3 **a** Micrograph of a *G. margarita* spore and the directions of the H^+ and Cl^- fluxes on germinated and non-germinated status. *GTE* corresponds to the region of germ tube emergence and *OGTE* to the region opposite to germ tube emergence. **b,c** H^+ and Cl^- ion flux profile in two spore zones using a H^+ and Cl^- specific vibrating probe (*error bars* average ± SE)

G. margarita germ tubes was acidic at the apical region, increasing to more alkaline values in the subapical region and regressing to neutral values in greater tip distance.

The hyphal tip was also shown to be susceptible to the cytoskeleton inhibitor Latrunculin A, which inhibits actin polymerization. The cytoskeleton of AM fungi is known to contain microtubules and microfilaments (Astrom et al. 1994), and beta tubulin has been shown to be expressed in germinating spores (Franken et al. 1997). From these results, it seems likely that the cytoplasm acidification and tip H^+ flux should be related to the presence of acidic small vesicles that could be delivered in the hyphae apex directed by the apical cytoskeleton network. The content of these vesicles could include acidic lytic enzymes, which could be involved in the growth and/or root penetration processes, after exocytosis to the extracellular clef at the tip.

H^+-ATPase activity is regulated by an autoinhibitory domain in the C-terminal region of the enzyme (Palmgren et al. 1991). In plants, the 14-3-3 protein interacts with the C-terminal of H^+-ATPase and acts as a positive modulator of the complex (DeBoer 1997; Bunney et al. 2002; Finnie et al. 2002). In particular, many of the cell's ion pumps and channels are either directly or indirectly modulated by 14-3-3

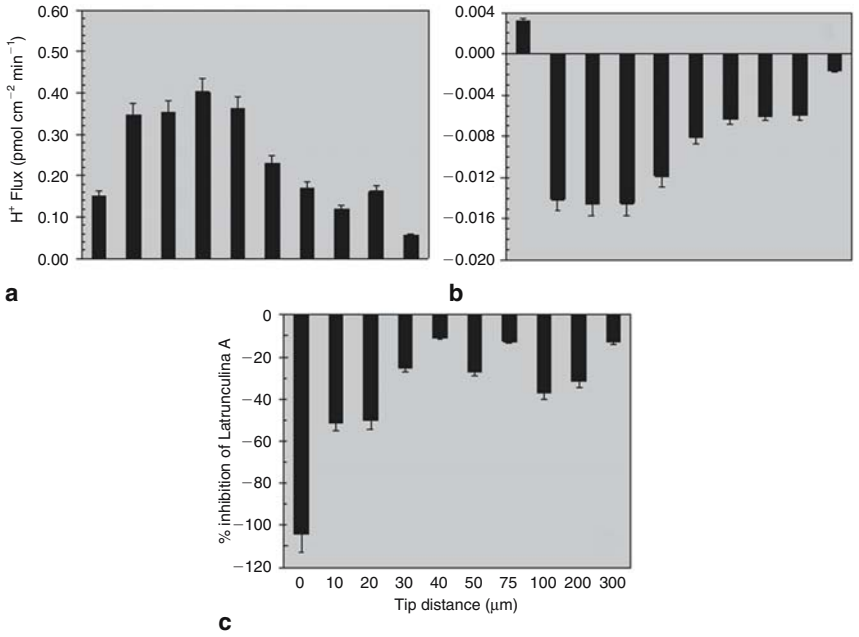

Fig. 4 **a** Proton flux profile in *G. margarita* hyphae obtained by H^+ -selective vibrating probe, 7 days after germination in M medium. **b** H^+ fluxes in the presence of 5 μM orthovanadate. The negative values correspond to influx and positive to efflux. **c** Percentage of H^+ efflux inhibition after treatment of 50 nM Latrunculin A, a cytoskeleton inhibitor (depolimerase actin microfilaments), showing a strong sensitivity of the H^+ flux at the hyphal tip and a differential action mode is observed in the rest of hyphal zones (*error bars* average±SE)

proteins (Bunney et al. 2002). Recently, a 14-3-3 protein encoding gene from *Glomus intraradices* (Gi14-3-3) was isolated by Porcel et al. (2006). It was highly expressed under drought stress suggesting that Gi14-3-3 gene may be involved in the adaptative strategies of this AM fungus to adverse conditions. In this specific case, the activation of the fungal P-type H^+-ATPase by the 14-3-3 protein could be important in maintaining the ionic gradient favorable to water and solutes entry into and retention in the fungal cells during the stress.

Although, molecular approaches have confirmed the expression of P-type H^+-ATPase genes in the AM fungi (Franken et al. 1997; Ferrol et al. 2000; Requena et al. 2003; Corradi et al. 2004), there is still a lack of information on the dynamics of the H^+ pumps placement along the hyphae. The difficulties in using Green Fluorescence Protein (GFP) reporter gene in AMF is due the background fluorescence levels within AMF being high, which decreases the sensitivity of this system (Forbes et al. 1998). Recently, a method to use fluorescent marker expression in an AM fungus driven by arbuscular mycorrhizal promoters was published by Helber and Requena (2008). It could be adjusted as an alternative to measure "in vivo" PM H^+-ATPase expression in AM fungal hyphae and their responses to root factors (Helber and Requena, 2008). An alternative approach would be to either delete (null

mutation) or overexpress a specific fungal isoform of the fungi H^+-ATPase and measure the changes in the hyphal H^+ fluxes, but so far such evidence is still a long way down the road.

4 Electrophysiological Responses as a Component of Signaling Plant–fungus: A Possible pH-sensing Mechanism

Data from extracellular ion fluxes and transmembrane electric potential differences (Em) in *G. margarita* performed in quite similar assay conditions have indicated that the germ tubes of AM fungi are weakly polarized during presymbiosis (without any root factor). For instance, measurements of total membrane electric potential carried out by Ayling et al. (2000) revealed Em values of germ tubes of *G. margarita* around −40 mV, a value much lower than −200 mV in *Neurospora crassa* (Miller et al. 1990), −160 mV in *Achlya bisexualis* (Kropf et al. 1984) and −132 mV in pollen tubes (Weisenseel et al. 1975). Likewise, Berbara et al. (1995), using a one-dimension vibrating voltage probe, measured small total currents (~20 μA cm^{-2}) at the tip of germ tubes of *G. margarita*, which were also much weaker than that found in clover roots and in other fungi (Ayling et al. 2000). Such characteristics could be related to the AM fugal H^+ pumping capacity, since the magnitude of the H^+ fluxes detected in the AM hyphae was also much lower than that observed in polarized plant cells, such as pollen tubes (Feijó et al. 1999). It indicates that the germ tubes of AM fungi are weakly polarized in the presymbiosis without any root factor present. It is possible that there is a tight control of the spore reserves mobilization to keep just a basal ATP-consuming metabolism during the presymbiotic development. In agreement with this possibility it was found that- in the presence of host roots exudates- this scenario changes as a burst in the activation of the ion fluxes is observed.

Electrophysiological studies during the presymbiosis of *G. margarita* demonstrated that electrical membrane potential (Em) becomes hyperpolarized when plant root extracts were added to the medium (Ayling et al. 2000). Actually, root exudates and CO_2 derived from host roots in the vicinity of the AM hyphae have been shown to stimulate the AM hyphal growth at the early stages of fungal development (Bécard and Piché 1989; Nair et al. 1991; Bécard et al. 1992; Chabot et al. 1992). Most root exudates contain several active compounds, such as peptides/proteins and flavonoids (Harrisson 2005), but the identity and function of most of them are not yet fully known. Although flavonoid derivatives can influence the initial stages of the fungal life cycle, experiments with flavonoid-deficient mutants of maize indicate that they are not essential for the development of the AM symbiosis as previously believed (Bécard et al. 1995; Buee et al. 2000; Harrison 2005). Signaling molecules exuded from host roots are often called “branching factors (BFs)” that induce extensive hyphal branching in AM fungi (Buee et al. 2000). Recently, using a simple and elegant approach, Akiyama et al. (2005) purified the BF from root exudates of *Lotus japonicus*, and discovered that the BF is a strigolactone, which is very effective at extremely low concentrations (10^{-13} M).

Besserer et al. (2006) showed a response of AM fungal hyphae to strigolactones similar to those reported previously and involving changes in mitochondrial density and respiration. Despite being part of the root exudate, no electrophysiological data is available about the separated effects of strigolactone or flavonoids in AM fungal hyphae during presymbiosis.

In parallel, cytochemical studies showed a stimulation on fungal plasma membrane H^+-ATPases and Pi uptake after treatment by root exudates and CO_2 (Lei et al. 1991). Assuming the notion that for Pi uptake is necessary for the activity of P-type H^+-ATPases, it is crucial to establish the proton-motive force required for cotransport H^+/PO^{4-} and also for other solutes (Mimura 1995; Morsomme and Boutry 2000). Pi uptake, on the other hand, is needed to uphold the ATP synthesis, which in turn, is indispensable to feed the H^+-ATPase activity. It appears that when an AM fungi can sense the signals of a nearby host root, a cascade of events is activated, finally inducing the hyphal branching and growth towards the fastest interaction. Evidence suggests that the modulation of the hyphal H^+ flux and cytoplasmic pH exerts a key role in this phenomenon.

In agreement with the data of H^+ efflux, Jolicoeur et al. (1998) reported the intracellular pH profile in *Gigaspora margarita* hyphae is more alkaline in the subtropical region, when the fungi were growing in the presence of (but without any direct contact with) *Daucus carota* roots. More recently, Requena et al. (2003) analyzed the expression of two isoforms (GmPMA1 and GmHA5) of the plasma membrane H^+-ATPase from *Glomus mosseae*. They found that GmPMA1 was highly expressed during fungal presymbiotic development, whereas the GmHA5 transcript was induced at the appressorium stage when the fungus enters in contact with the host root (Requena et al. 2003).

On the other hand, it is likely that AM fungi can also exudate active molecules that can regulate the H^+-ATPase activity of the plant cell. It is well known that 14-3-3 proteins are the main physiologically relevant regulators of the plant H^+-ATPase activity and a number of ion channels. The mechanism involves protein phosphorilation elicited by different stimuli, followed by the binding of 14-3-3 proteins to auto-regulatory domains resulting in the pump/channel activation (Morsomme and Boutry 2000). The activation complex 14-3-3 protein–proton pump is highly stabilized in the presence of the fungal toxin fusicoccin (Bunney et al. 2002). Moreover, there is evidence that H^+-ATPase dephosphorylation can also induce pump activation in response to other fungal elicitors (Veraestrella et al. 1994). In this study, pathogenic fungal elicitors led to a 4-fold increase in ATPase activity of the plasma membrane, and this increase could be prevented by addition of the phosphatase inhibitor okadaic acid. Although these elicitors are synthesized by pathogenic fungi, it is possible that AM fungi also produce some molecules with similar stimulatory capacity, since both pathogenic and symbiotic hyphal depend on their penetration capacity through the plant cell wall. In accordance, we have described an activation of maize root proton pumps induced by mycorrhization with either *Glomus clarum* or *Gigaspora margarita* fungi (Ramos et al. 2005). It was postulated that the apoplast acidification resultant from such activation could promote the activity of the lytic enzymes responsible by cell wall plasticity,

therefore facilitating the hyphae penetration. The C-terminal auto-regulatory domain of the fungal P-type H^+-ATPase is very different from its counterpart of the plant pump (Bunney et al. 2002) and, therefore, the fungal and plant enzymes should not be regulated in a same manner. Tamasloukht et al. (2003) showed that root exudates induced very fast response in gene expression (0.5–1 h), consumption of oxygen (3 h), morphology (5 h), and changed the number and shape of fungal mitochondria of *Gigaspora rosea* and *Glomus intraradices*. In interesting work done by Lanfranco et al. (2005), they isolated and identified a Cu/Zn superoxide dismutases (SODs) expressed in germinated spores of *Gigaspora margarita*. They reported that the expression of this enzyme is also enhanced after treatment of host root exudates. A higher transcript level of "GmarCuZnSOD" could reflect an enhanced capacity to remove the excess of ROS resulted from the burst of respiration rate previously reported (Tamasloukht et al. 2003). For the protection against ROS, "CuZnSOD" might be involved in root exudate perception (Lanfranco et al. 2005).

Furthermore, the pattern of fungal response not only to root exudates, but also to CO2 concentration, seems to define the transition from the asymbiotic to pre-symbiotic developmental stage. A synergistic action of CO_2 with root exudates on growth stimulation was in fact described for *Gigaspora rosea* (Bécard and Piché 1989). Treatment of AM fungus with 1% CO_2 induced a highly oscillatory profile for the H^+ flux in function of time, but with a clear predominance of H^+ effluxes (Fig. 5). Taken together this data raises an interesting relationship between the AM fungi electrophysiological responses and their developmental metabolism.

In the absence of root factors the AM fungi have a basal metabolism, as demonstrated by low values of H^+ fluxes, electrical currents and membrane potential. Therefore, when root signals including CO_2 are recognized and processed by AM fungal cells they may activate their metabolism, and could sustain the hyphal growth and branching, characteristics of the presymbiotic phase. Tamasloukht et al.

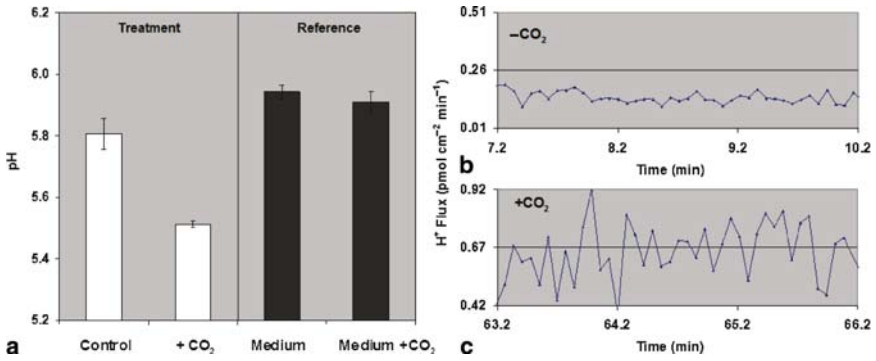

Fig. 5 **a** Effect of the treatment with 1% CO2 on subapical hyphal surface pH. The M medium was exchanged more than 5 times in order remove medium acidification promoted by CO2. **b,c** The incubation of germinated spores of *G. margarita* for 3 h induced extracellular hyphal H^+ fluxes and its oscillatory component. The *graphs* represents oscillation in intervals of 3 min and the same scale was used for H^+ flux. *Error bars* 95% confidence intervals (SE)

(2003) raised an interesting explanation for the AM fungal response to CO_2. They speculated that germinating spores of AM fungi exhibit a low respiratory activity during presymbiotic growth in order to minimize carbon consumption from their own resources (trehalose, glycogen, and lipids), and the incorporation of additional carbon even in the form of CO_2 could represent a new source of energy, since it is that CO_2 dark fixation which takes place in *Glomus intraradices* germ tubes (Bago et al. 1999a; Lammers et al. 2005).

Plant receptor system can recognize "nonself" molecules characteristic of fungi and bacteria with a high degree of specificity, and some of them show rapid desensitization. The balance between the activity of proton pumps and the secondary transport systems responsible for H^+ fluxes are believed to be the major carriers of electrical currents across the plasma membrane. The orchestrated regulation of these systems can generate electrical signals, which modulate fungal membrane potential and electrical currents triggering early events important for the symbiotic interaction. Indeed, numerous physiological effects of electrical signaling have been discovered in the past two decades (Fromm and Lautner 2007). Electrical signals are able to rapidly transmit information over long distances. Sbrana and Giovannetti (2005) using an interesting approach reported that AM fungal hyphae grow directionally towards host roots. They proposed that this chemotropism could be regarded as a second mechanism (the first being the hyphal-branching) promoting the recognition and interaction amongst the symbionts. This phenomenon has proved to be host-specific, since treatments with nonhost plants and dead plants did not induced any chemotaxis (Sbrana and Giovannetti 2005). The electrical phenomena could also act as a synergistic effect of the chemotaxis-tropism (Boller 1995), since the elongating region of roots produces high electrical current and fields (due the major activity of P-type H^+-ATPases), which are known to induce hyphal orientation and branching (Weisenseel et al. 1975; Gow 1989; Sbrana and Giovannetti 2005; Fromm and Lautner 2007). However, the electrical signals are carried out mainly by ionic fluxes across the plasma membrane, and when the stimulus is sufficiently big to depolarize the membrane an action potential is generated (Fromm and Lautner 2007). Such membrane depolarization could be elicited by root exudates, modulating the AM fungal cytosolic pH, H^+ efflux and membrane potential. The nature of the chemotropic signal remains to be identified, but strigolactone seems to be one of the strongest possibilities. We propose that electrical signals are important in the long distance signaling, which can be seen as one primary component of the overall response.

5 Ions Play in the AM Signaling? Going to Symbiosis!

Up to now, there are just a few reports on the ion dynamics characterization of AM hyphae. In stark contrast, however, the most characterized phenomenon is the phosphate metabolism (Viereck et al. 2004; Fitter 2006). Calcium fluxes during the presymbiosis are well characterized for the effects of Nod factors in root hairs of

legume, but their physiological significance remains speculative during AM symbiosis. The first work involving Ca^{2+} dynamic and fungal factors was realized by Navazio et al. (2006). They used a Ca^{2+} bioluminescent indicator (aequorin) and found fast and transient Ca^{2+} waves induced by addition of fungal diffusible factor (molecules <3 kDa) released by the AM fungus *Gigaspora margarita*. After perception of the molecule by soybean culture cells, a Ca^{2+} -signal was rapidly induced (Navazio et al. 2006). Therefore, diffusible factor receptors appear to work differently from the Nod factor system. Using this approach, they could not detect any Ca^{2+} spiking in host cell cultures.

Besides Ca^{2+}, H^+ ions have also been recognized as important regulators of the cell growth and development in plant and fungal cells (Felle 2001; Hetherington and Brownlee 2004). Fungi have been the subject of contradictory reports about the cytosolic pH: an alkaline gradient has been reported in the growing hyphae of *Neurospora crassa* (Robson et al. 1996), whereas a cytoplasm pH gradient has been observed in *Gigaspora margarita* (Jolicoeur et al. 1998). However, both groups provided evidence for a role of cytosolic pH dynamic in the regulation of hyphal growth. Proton pumps are the primary energetic mechanism for membrane transport in most cells (Sondergaard et al. 2004; Baekgaard et al. 2005), and notably in AM fungal hyphae and root cells. The activity of the H^+-ATPase appears to be important to the polarized growth of AM fungi, as exposure to the P-type pump inhibitor, orthovanadate (Ramos et al. 2006) or dietilbestrol (Lei et al. 1991), impaired the hyphal growth. Previously, it was found that orthovanadate enters cells via the phosphate transport system and inhibits the growth of the *Neurospora crassa* fungi (Bowman 1983). Therefore, the H^+ infflux observed in Fig. 4 in different regions of the fungal hyphae in response to the orthovanadate (VO^{-4}) could be interpreted as the cotransport H^+/VO^{-4}.

In the light of the data discussed during this review, a hypothetical mechanism based on ion signaling between plant roots and AM fungi can be proposed (Fig. 6). Briefly, the communication between the symbionts starts before any physical contact between fungus and plant. Root factors released by host plant roots, namely strigolactone, proteins, CO_2 and extracellular proton fluxes, are recognized by specific receptors in the fungal plasma membrane (Lambais 2006; Requena et al. 2007), and these factors trigger a signal transduction cascade involving:

1. Activation of specific fungal symbiotic genes by interaction/modification of membranar proteins by Gin1 (Requena et al. 2002) which transmits the signal towards the nucleus (Requena et al. 2007).
2. This primary gene-induction could include 14-3-3 proteins, kinases and phosphatases.
3. These will increase the activity of P-type H^+-ATPase.
4. As a consequence a localized increase in the intracellular pH is caused (Jolicoeur et al. 1998).
5. The electrochemical gradient generated will energize secondary transporters a and voltage-dependent channels, inducing the uptake of Ca^{2+}, Cl^-, K^+, glucose, phosphate and other metabolites.

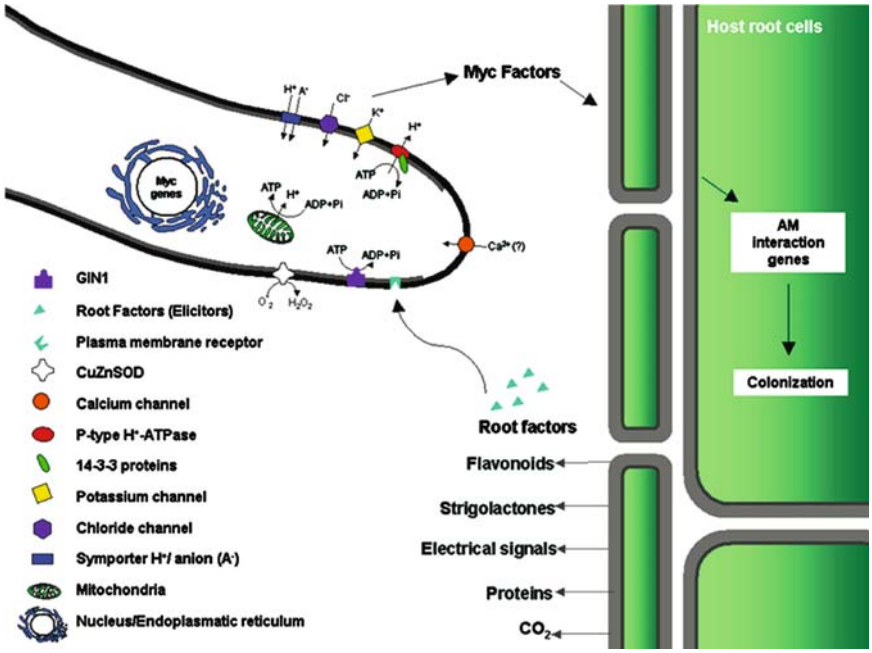

Fig. 6 A basic model of the fungal responses to the host signals

6. As the ATP demand will increase, the upregulation of genes related to mitochondrial metabolism (30 min) and respiratory rate (3 h) will increase the ATP availability and the cell metabolism in general (Tamasloukht et al. 2003).
7. A "GmarCuZnSOD" (Lanfranco et al. 2005) and likely other anti-oxidative enzymes could be activated, in order to overcome the ROS-inactivating system related to the host defense, providing the first contact between the host roots and AM fungus, that differentiate into the appressorium and colonize the root cortex. Removal of the excess of ROS produced during high respiration rate had been proposed (Lanfranco et al, 2005; Tamasloukht et al. 2003).
8. In response, the root would perceive the hyphae and trigger the expression of specific AM interaction genes responsible for symbiosis.

Such a model, while incorporating the available evidence remains speculative in most steps, but provides a useful framework for hypothesis testing in many respects, which we are in the process of experimentally addressing.

6 Conclusions

Given the cytosolic pH dynamics and the corresponding H^+ efflux patterns described above, it seems likely that a specific H^+-ATPase regulation is present mainly in the plasma membrane at subapical region. Clearly, further data on the

mechanism of action of signaling molecules such as strigolactones over the signal transduction and ion uptake in AM fungi will be very important to improve our understanding of the molecular bases of the mycorrhization process. Future studies are necessary in order to provide basic knowledge of the ion signaling mechanisms and their role on the response of very important molecules playing at the early events of AM symbiosis.

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References

Akiyama K, Hayashi H (2006) Strigolactones: chemical signals for fungal symbionts and parasitic weeds in plant roots. Ann Bot 97:925–931

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Alessandro C. Ramos, Arnoldo R. Façanha and José A. Feijó. (2008) Proton (H^+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi. New Phytologist 178:1, 177–188

Astrom H, Giovannetti M, Raudaskoski M (1994) Cytoskeletal components in the arbuscular mycorrhizal fungus *Glomus mosseae*. Mol Plant Microb Interact 7:309–312

Ayling SM, Smith SE, Smith FA (2000) Transmembrane electric potential difference of germ tubes of arbuscular mycorrhizal fungi responds to external stimuli. New Phytol 147:631–639

Baekgaard L, Fuglsang AT, Palmgren MG (2005) Regulation of plant plasma membrane H^+- and Ca^{2+}-ATPases by terminal domains. J Bioenerg Biomembr 37:369–374

Bago B, Zipfel W, Williams RM, Chamberland H, Lafontaine JG, Webb WW, Piché Y (1998) In vivo studies on the nuclear behavior of the arbuscular mycorrhizal fungus *Gigaspora rosea* grown under axenic conditions. Protoplasma 203:1–15

Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y (1999a) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Bago B, Zipfel W, Williams RM, Piché Y (1999b) Nuclei of symbiotic arbuscular mycorrhizal fungi as revealed by *in vivo* two-photon microscopy. Protoplasma 209:77–89

Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–957

Bago B, Pfeffer PE, Zipfel W, Lammers P, Shachar-Hill Y (2002a) Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi. Metabolism and transport in AM fungi. Plant Soil 244:189–197

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002b) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Barr R (1991) The possible pole of redox-associated protons in growth of plant–cells. J Bioenerg Biomembr 23:443–467

Bartnicki-Garcia S, Bracker CE, Gierz G, Lopez-Franco R, Lu HS (2000) Mapping the growth of fungal hyphae: orthogonal cell wall expansion during tip growth and the role of turgor. Biophys J 79:2382–2390

Bécard G, Piché Y (1989) Fungal growth-stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Bécard G, Douds DD, Pfeffer PE (1992) Extensive invitro hyphal growth of vesicular-arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. Appl Environ Microbiol 58:821–825

Bécard G, Taylor LP, Douds DD, Pfeffer PE, Doner LW (1995) Flavonoids are not necessary plant signal compounds in arbuscular mycorrhizal symbioses. Mol Plant Microb Interact 8:252–258

Berbara RLL, Morris BM, Fonseca H, Reid B, Gow NAR, Daft MJ (1995) Electrical currents associated with arbuscular mycorrhizal interactions. New Phytol 129:433–438

Besserer A, Puech-Pages V, Kiefer P, Gomez-Roldan V, Jauneau A, Roy S, Portais JC, Roux C, Bécard G, Sejalon-Delmas N (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. Plos Biol 4:1239–1247

Boavida LC, Vieira AM, Becker JD, Feijó JA (2005). Gametophyte interaction and sexual reproduction: how plants make a zygote. Int J Dev Biol. 49:615–632.

Boller T (1995) Chemoperception of microbial signals in plant cells. Annu Rev Plant Physiol Plant Mol Biol 46:189–214

Bowman BJ (1983) Vanadate uptake in *Neurospora crassa* occurs via phosphate transport system II. J Bacteriol 153:286–291.

Buee M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microb Interact 13:693–698

Bunney TD, van den Wijngaard PWJ, de Boer AH (2002) 14-3-3 protein regulation of proton pumps and ion channels. Plant Mol Biol 50:1041–1051

Campanoni P, Blatt MR (2007) Membrane trafficking and polar growth in root hairs and pollen tubes. J Exper Bot 58:65–74

Cárdenas L, Feijó JA, Kunkel JG, Sanchez F, Holdaway-Clarke T, Hepler PK, Quinto C (1999) Rhizobium Nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs. Plant J 19:347–352

Chabot S, Belrhlid R, Chenevert R, Piché Y (1992) Hyphal growth promotion invitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker and Hall, by the activity of structurally specific flavonoid compounds under CO2 enriched conditions. New Phytol 122:461–467

Cheung AY, Wu HM (2007) Structural and functional compartmentalization in pollen tubes. J Exper Bot 58:75–82

Cleland RE (1993) Role of acidification in cell-wall expansion. J Cell Biochem:4–4

Corradi N, Kuhn G, Sanders IR (2004) Monophyly of beta-tubulin and H^+-ATPase gene variants in *Glomus intraradices*: consequences for molecular evolutionary studies of AM fungal genes. Fungal Genet Biol 41:262–273

Crampin H, Finley K, Gerami-Nejad M, Court H, Gale C, Berman J, Sudbery P (2005) Candida albicans hyphae have a Spitzenkorper that is distinct from the polarisome found in yeast and pseudohyphae. J Cell Sci 118:2935–2947

DeBoer B (1997) Fusicoccin - a key to multiple 14-3-3 locks? Trends Plant Sci 2:60–66

Feijó JA, Malho R, Obermeyer G (1995) Ion dynamics and its possible role during in-vitro pollen germination and tube growth. Protoplasma 187:155–167

Feijó JA, Sainhas J, Hackett GR, Kunkel JG, Hepler PK (1999) Growing pollen tubes possess a constitutive alkaline band in the clear zone and a growth-dependent acidic tip. J Cell Biol 144:483–496

Feijó JA, Sainhas J, Holdaway-Clarke T, Cordeiro MS, Kunkel JG, Hepler PK (2001) Cellular oscillations and the regulation of growth: the pollen tube paradigm. Bioessays 23:86–94

Feijó JA, Costa SS, Prado AM, Becker JD, Certal AC (2004) Signalling by tips. Curr Opin Plant Biol 7:589–598

Felle HH (2001) pH: signal and messenger in plant cells. Plant Biol 3:577–591

Felle HH, Herrmann A, Hanstein S, Huckelhoven R, Kogel KH (2004) Apoplastic pH signaling in barley leaves attacked by the powdery mildew fungus *Blumeria graminis* f. sp hordei. Mol Plant Microb Interact 17:118–123

Ferrol N, Barea JM, Azcon-Aguilar C (2000) The plasma membrane H^+-ATPase gene family in the arbuscular mycorrhizal fungus *Glomus mosseae*. Curr Genet 37:112–118

Finnie C, Andersen CH, Borch J, Gjetting S, Christensen AB, de Boer AH, Thordal-Christensen H, Collinge DB (2002) Do 14-3-3 proteins and plasma membrane H^+-ATPases interact in the barley epidermis in response to the barley powdery mildew fungus? Plant Mol Biol 49:137–147

Fitter AH (2006) What is the link between carbon and phosphorus fluxes in arbuscular mycorrhizas? A null hypothesis for symbiotic function. New Phytol 172:3–6

Forbes PJ, Millam S, Hooker JE, Harrier LA (1998) Transformation of the arbuscular mycorrhiza *Gigaspora rosea* by particle bombardment. Mycol Res 102:497–501

Franken P, Lapopin L, MeyerGauen G, Gianinazzi-Pearson V (1997) RNA accumulation and genes expressed in spores of the arbuscular mycorrhizal fungus, *Gigaspora rosea*. Mycologia 89:293–297

Fromm J, Lautner S (2007) Electrical signals and their physiological significance in plants. Plant Cell Environ 30:249–257

Gianinazzi-Pearson V (1996) Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell 8:1871–1883

Giovannetti M, Sbrana C, Avio L, Citernesi AS, Logi C (1993) Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during preinfection stages. New Phytol 125:587–593

Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal recognition responses to host derived signals by arbuscular mycorrhizal fungi. New Phytol 133:65–71

Gow NAR (1989) Circulating ionic currents in microorganisms. Adv Microb Physiol 30:89–123

Harris SD (2006) Cell polarity in filamentous. In: International review of cytology - a survey of cell biology, vol 251, pp 41–77

Harris SD, Momany M (2004) Polarity in filamentous fungi: moving beyond the yeast paradigm. Fungal Genet Biol 41:391–400

Harris SD, Read ND, Roberson RW, Shaw B, Seiler S, Plamann M, Momany M (2005) Polarisome meets Spitzenkorper: microscopy, genetics, and genomics converge. Eukaryotic Cell 4:225–229

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Helber N, Requena N (2008) Expression of the fluorescence markers DsRed and GFP fused to a nuclear localization signal in the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 177:537–548

Hepler PK (2005) Calcium: a central regulator of plant growth and development. Plant Cell 17:2142–2155

Hepler PK, Vidali L, Cheung AY (2001) Polarized cell growth in higher plants. Annu Rev Cell Dev Biol 17:159–187

Hetherington AM, Brownlee C (2004) The generation of Ca^{2+} signals in plants. Annu Rev Plant Biol 55:401–427

Holdaway-Clarke TL, Hepler PK (2003) Control of pollen tube growth: role of ion gradients and fluxes. New Phytol 159:539–563

Jolicoeur M, Germette S, Gaudette M, Perrier M, Bécard G (1998) Intracellular pH in arbuscular mycorrhizal fungi: a symbiotic physiological marker. Plant Physiol 116:1279–1288.

Kim YC, Wikstrom M, Hummer G (2007) Kinetic models of redox-coupled proton pumping. Proc Natl Acad Sci USA 104:2169–2174

Kropf DL, Caldwell JH, Gow NAR, Harold FM (1984) Trans-cellular ion currents in the water mold Achlya - aminoacid proton symport as a mechanism of current entry. J Cell Biol 99:486–496

Lambais MR (2006) Unraveling the signaling and signal transduction mechanisms controlling arbuscular mycorrhiza development. Sci Agric 63:405–413

Lammers PJ, Jun J, Abubaker J, Arreola R, Gopalan A, Bago B, Hernandez-Sebastia C, Allen JW, Douds DD, Pfeffer PE, Shachar-Hill Y (2001) The glyoxylate cycle in an arbuscular mycorrhizal fungus. Carbon flux and gene expression. Plant Physiol 127:1287–1298

Lanfranco L, Novero M, Bonfante P (2005) The mycorrhizal fungus *Gigaspora margarita* possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. Plant Physiol 137:1319–1330

Lei J, Bécard G, Catford JG, Piché Y (1991) Root factors stimulate P32 uptake and plasmalemma ATPase activity in vesicular arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 118:289–294

Maia LC, Kimbrough JW (1994) Ultrastructural studies on spores of *Glomus intraradices*. Int J Plant Sci 155:689–698

Maia LC, Kimbrough JW, Benny GL (1994) Ultrastructure of Spore Germination in *Gigaspora albida* (Glomales). Mycologia 86:343–349

Miller AJ, Vogg G, Sanders D (1990) Cytosolic calcium homeostasis in fungi - roles of plasma membrane transport and intracellular sequestration of calcium. Proc Natl Acad Sci USA 87:9348–9352

Miller AL, Gow NAR (1989) Correlation between profile of ion current circulation and root development. Physiol Plant 75:102–108

Miller AL, Smith GN, Raven JA, Gow NAR (1991) Ion currents and the nitrogen status of roots of *Hordeum vulgare* and non-nodulated *Trifolium repens*. Plant Cell Environ 14:559–567

Mimura T (1995) Homeostasis and transport of inorganic phosphate in plants. Plant Cell Physiol 36:1–7

Morsomme P, Boutry M (2000) The plant plasma membrane H^+-ATPase: structure, function and regulation. Biochim Biophys Acta Biomembr 1465:1–16

Nair MG, Safir GR, Siqueira JO (1991) Isolation and identification of vesicular-arbuscular mycorrhiza-stimulatory compounds from clover (*Trifolium repens*) roots. Appl Environ Microbiol 57:434–439

Navazio L, Moscatiello R, Genre A, Novero M, Baldan B, Bonfante P, Mariani P (2006) A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells. Plant Physiol DOI:10.1104.

Palmgren MG (2001) Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. Annu Rev Plant Physiol Plant Mol Biol 52:817–845

Palmgren MG, Sommarin M, Serrano R, Larsson C (1991) Identification of an autoinhibitory domain in the c-terminal region of the plant plasma membrane H^+-ATPase. J Biol Chem 266:20470–20475

Porcel R, Aroca R, Cano C, Bago A, Ruiz-Lozano JM (2006) Identification of a gene from the arbuscular mycorrhizal fungus glomus intraradices encoding for a 14-3-3 protein that is up-regulated by drought stress during the AM symbiosis. Microb Ecol 52:575–582.

Portillo F (2000) Regulation of plasma membrane H^+-ATPase in fungi and plants. Biochim Biophys Acta Rev Biomembr 1469:31–42

Ramos AC, Martins MA, Facanha AR (2005) ATPpase and Pyrophosphatase activities in corn root microsomes colonized with arbuscular mycorrhizal fungi. Rev Brasil Ciencia Solo 29:207–213

Ramos AC, Façanha AR, Feijó JA (2006) A H^+ ion flux signature for the hyphal asymbiotic development of the am fungus *Gigaspora margarita*. International Congress of Mycorrhiza (ICOM 5), Granada, Spain.

Ramos AC, Façanha AR, Feijó JA (2008) Proton (H^+) flux signature for the presymbiotic development of the arbuscular mycorrhizal fungi. New Phytologist 178:1, 177–188

Rayle DL, Cleland RE (1992) The acid growth theory of auxin-induced cell elongation is alive and well. Plant Physiol 99:1271–1274

Remy W, Taylor TN, Hass H, Kerp H (1994) 4-hundred-million-year-old vesicular-arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Requena N, Mann P, Hampp R, Franken P (2002) Early developmentally regulated genes in the arbuscular mycorrhizal fungus *Glomus mosseae*: identification of GmGIN1, a novel gene with homology to the C-terminus of metazoan hedgehog proteins. Plant Soil 244:129–139

Requena N, Breuninger M, Franken P, Ocon A (2003) Symbiotic status, phosphate, and sucrose regulate the expression of two plasma membrane H^+-ATPase genes from the mycorrhizal fungus *Glomus mosseae*. Plant Physiol 132:1540–1549

Requena N, Serrano E, Ocon A, Breuninger M (2007) Plant signals and fungal perception during arbuscular mycorrhiza establishment. Phytochemistry 68:33–40

Riquelme M, Bartnicki-Garcia S (2004) Key differences between lateral and apical branching in hyphae of Neurosora crassa. Fungal Genet Biol 41:842–851

Robson GD, Prebble E, Rickers A, Hosking S, Denning DW, Trinci APJ, Robertson W (1996) Polarized growth of fungal hyphae is defined by an alkaline pH gradient. Fungal Genet Biol 20:289–298

Saito K, Kuga-Uetake Y, Saito M (2004) Acidic vesicles in living hyphae of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. Plant Soil 261:231–237

Sbrana C, Giovannetti M (2005) Chemotropism in the arbuscular mycorrhizal fungus *Glomus mosseae*. Mycorrhiza 15:539–545

Scott DA, Docampo R (1998) Two types of H^+-ATPase are involved in the acidification of internal compartments in *Trypanosoma cruzi*. Biochem J 331:583–589

Seiler S, Plamann M (2003) The genetic basis of cellular morphogenesis in the filamentous fungus *Neurospora crassa*. Mol Biol Cell 14:4352–4364

Siqueira JO, Sylvia DM, Gibson J, Hubbell DH (1985) Spores, germination, and germ tubes of vesicular-arbuscular mycorrhizal fungi. Can J Microbiol 31:965–972

Smith SE, Read DJ, eds (1997) Mycorrhizal symbiosis. Academic, London

Sondergaard TE, Schulz A, Palmgren MG (2004) Energization of transport processes in plants. Roles of the plasma membrane H^+-ATPase. Plant Physiol 136:2475–2482

Sward RJ (1981a) The structure of the spores of *Gigaspora margarita* .2. Changes accompanying germination. New Phytol 88:661–669

Sward RJ (1981b) The structure of the spores of *Gigaspora margarita* .3. Germ-tube emergence and growth. New Phytol 88:667–669

Tamasloukht M, Sejalon-Delmas N, Kluever A, Jauneau A, Roux C, Bécard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. Plant Physiol 131:1468–1478

Taylor TN, Remy W, Hass H, Kerp H (1995) Fossil arbuscular mycorrhizae from the early Devonian. Mycologia 87:560–573

Uetake Y, Kojima T, Ezawa T, Saito M (2002) Extensive tubular vacuole system in an arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 154:761–768

Veraestrella R, Higgins VJ, Blumwald E (1994) Plant defense response to fungal pathogens .2. G-protein-mediated changes in host plasma-membrane redox reactions. Plant Physiol 106:97–102

Viereck N, Hansen PE, Jakobsen I (2004) Phosphate pool dynamics in the arbuscular mycorrhizal fungus *Glomus intraradices* studied by *in vivo* P-31 NMR spectroscopy. New Phytol 162:783–794

Vissenberg K, Feijó JA, Weisenseel MH, Verbelen JP (2001) Ion fluxes, auxin and the induction of elongation growth in *Nicotiana tabacum* cells. J Exper Bot 52:2161–2167

Weisenseel MH, Nuccitelli R, Jaffe LF (1975) large electrical currents traverse growing pollen tubes. J Cell Biol 66:556–567

Xing T, Higgins VJ, Blumwald E (1996) Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+-ATPase. Plant Cell 8:555–564

Zonia L, Cordeiro S, Tupy J, Feijó JA (2002) Oscillatory chloride efflux at the pollen tube apex has a role in growth and cell volume regulation and is targeted by inositol 3,4,5,6-tetrakisphosphate. Plant Cell 14:2233–2249

Arbuscular Mycorrhiza in Metal Hyperaccumulating Plants

Marjana Regvar(✉), Katarina Vogel-Mikuš

1 Introduction

The presence of specific vegetation connected to "black stones" (ultramafic rocks) was already noted in the sixteenth century, but the unusual metal accumulation in plants was reported in *Alyssum bertolonii* only in 1948. Since then, metal hyperaccumulating plants have been found among the families of Acanthaceae, Apocynaceae, Asteraceae, Amaranthaceae, Brassicaceae, Buxaceae, Caryophyllaceae, Celastraceae, Clusiaceae, Commelinaceae, Cunoniaceae, Cyperaceae, Dichapetalaceae, Euphorbiaceae, Fabaceae, Flacourtiaceae, Lamiaceae, Meliaceae, Myrtaceae, Ochnaceae, Plumbaginaceae, Poaceae, Polygonaceae, Proteaceae, Pteridaceae, Rubiaceae, Sapotaceae, Stachousiaceae, Scrophulariaceae, Tiliaceae, Turneraceae, Velloziaceae and Violaceae (Brooks 1998; Reeves 2006). Until recently (Turnau and Mesjasz-Przybylowicz 2003; Vogel-Mikuš et al. 2005), they were widely accepted as non-mycorrhizal (Leyval et al. 1997; Pawlowska et al. 2000). Considerable scepticism exists about the functional importance of arbuscular mycorrhizae (AM) in highly tolerant hyperaccumulating plants, but nevertheless its functionality was recently confirmed (Vogel-Mikuš et al. 2006). Phytoremediation is the cheapest among the techniques currently developed for metal remediation (Glass 2000). Considerable scientific efforts are therefore being directed into the improvement of the efficiency of phytoremediation using biotechnology tools. Mycorrhizal inoculation with its functional significance for plant fitness (Vogel-Mikuš et al. 2006) could contribute significantly to the success of phytoremediation. Obvious obstacles in plant properties, however, limit the success of improvements to date (Salt et al. 1995). This chapter focuses on the general properties and worldwide occurrence of metal hyperaccumulating plants, the discovery of AM symbiosis and its possible roles in these highly metal-tolerant plant species.

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2 Metal Hyperaccumulations: State of the Art

2.1 Metal Hyperaccumulation: Terminology and Definitions

Metalliferous soils with abnormally high concentrations of some of the elements that are normally present as minor constituents (200–2,000 mg kg^{-1}, e.g., Mn) or trace constituents (0.01–200 mg kg^{-1}, e.g., Zn, Cu, Ni, Cr, Pb, As, Co, Se, Cd) vary widely in their effects on plants. These effects depend on plant species, the particular assemblage of enriched elements, and the physical and chemical characteristics of the soil (Reeves 2006).

There are some extreme examples of soils being toxic to almost all species of higher plants, whereas in other cases a characteristic flora of metal-tolerant species may develop (Reeves 2006). In plants that are able to survive in metal-polluted environments, three basic metal uptake and tolerance strategies of *exclusion*, *indication* and *accumulation* evolved for coping with excess metals (Baker 1981, 1987). In metal *excluders,* low to moderate metal concentrations are maintained in the tissues over a wider range of total soil metal concentrations, until critical concentrations cause damage to the exclusion mechanisms, resulting in inadvertent metal uptake. The relationship between soil and plant metal concentrations can thus be described by an exponential curve (Fig. 1) (Baker 1981). These plant species stabilize the metals present in soil and efficiently reduce their transfer through the food chain; therefore, they may be of particular interest for remediation of moderately polluted urban areas. In metal *indicators* the uptake and transport of metals to the shoot are regulated so that tissue concentrations reflect soil metal concentrations; therefore, the relationship between soil and plant metal concentrations can be described by a linear curve (Fig. 1) (Baker 1981). Plant species with this property

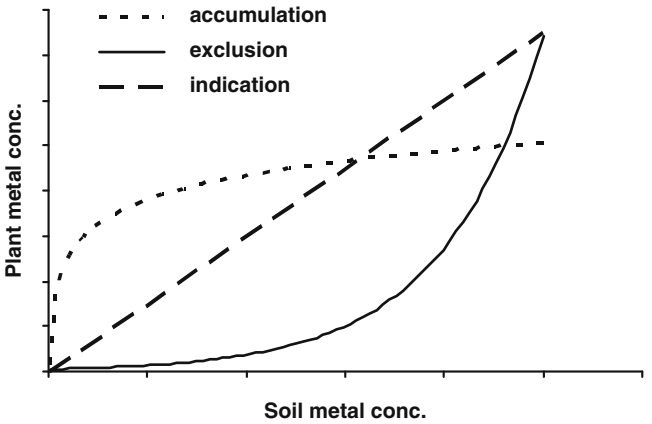

Fig. 1 The relationship between soil and plant metal concentrations in metal accumulator, indicator and excluder plant species (adopted after Baker 1981)

do give some indication of the plant-available (phytoavailable) metal pool needed for risk assessments, but factors affecting the uptake of available metals in plants severely reduce the possibility of the development of plant species as suitable indicators of absolute metal bioavailability. Therefore, we are still in the situation of having to grow individual plant species in contaminated soils to assess what portion of the metal loading is phytoavailable (Naidu et al. 2003). Some plant species are characterized by their ability to accumulate very high concentrations of certain elements, far in excess of normal physiological requirements (if any), and far in excess of the levels found in the majority of other species tolerant of metalliferous soils (Baker 1981; Reeves 2006). In these *accumulator* plants, the relationship between soil and plant metal concentrations can therefore be described by a logarithmic curve (Fig. 1) (Baker 1981). The specific use of *hyperaccumulation* to denote a defined concentration (greater than 1,000 mg kg^{-1}) was first introduced by Brooks et al. (1977) in discussing Ni concentrations in species of *Homalium* and *Hybanthus* from various parts of the world. The Ni concentration threshold of >1,000 mg kg^{-1} in dry leaf tissues was selected because this was 100 to 1,000 times higher than that normally found in plants on soils not of ultramafic origin, and 10 to 100 times higher than that found in most other plants on Ni-rich ultramafic soils. The criterion is not entirely arbitrary, as it has been noted that in many ultramafic floras Ni concentrations of 100–1,000 mg kg^{-1} are quite rare, and accumulation to >1,000 mg kg^{-1} seems to represent a distinct form of plant response, implying some characteristic and unusual metabolic behavior that usually characterizes the species as a "hyperaccumulator" or as a "normal" plant on this basis (Reeves 2006).

To define Ni hyperaccumulation more precisely, Reeves (1992) suggested the following definition: a hyperaccumulator of Ni is a plant in which a Ni concentration of at least 1,000 mg kg^{-1} has been recorded in the dry matter of any aboveground tissues in at least one specimen growing in its natural habitat. This indicates that the classification should not be based on analyses of whole plants or subterranean plant parts, largely because of the difficulty of ensuring that the samples are free from soil contamination, but also because plants that immobilize metals in the root system and fail to translocate them further are of less interest for many purposes then those that actively accumulate metals in their aboveground tissues (Reeves 2006). In practice, however, (1) some specimens of a species may be found with >1,000 mg kg^{-1}, whereas others occur with <1,000 mg kg^{-1}, (2) plant tissues other than leaves (e.g., latex) can be found with very high metal concentrations, or (3) a species may take up high levels of a metal under artificial conditions, such as through massive metal-salt amendments to an experimental soil or a nutrient solution. According to Reeves (2006), plants qualify as hyperaccumulators under cases (1) and (2), but not under (3). The "forced" uptake of added metals from nutrient solution often leads to plant mortality or other interruptions of full plant development, therefore such experiments frequently have little relevance to the behaviour of naturally occurring metallophyte populations, even though they may be of interest for some phytoremediation strategies. Furthermore, a plant species that is never found as a hyperaccumulator in nature may hyperaccumulate when given the opportunity on a real metalliferous (but not artificially amended) soil. Such a

species might be described as a "facultative" hyperaccumulator, particularly if it can be shown to complete its life cycle successfully on such a soil (Reeves 2006).

According to the most commonly used definition, nowadays a plant is declared a hyperaccumulator when meeting the following three requirements:

1. The concentration of the metal in the shoot must be higher than 10,000 mg kg^{-1} for Zn and Mn, higher than 1,000 mg kg^{-1} for Al, As, Se, Ni, Co, Cr, Cu and Pb, and higher than 100 mg kg^{-1} for Cd (Baker and Brooks 1989; Reeves and Baker 2000; Reeves 2006).
2. The shoot to soil concentration ratio, termed the *bioaccumulation factor* (BAF = C_{shoot} /C_{soil}), must be higher than 1, stressing higher metal concentrations in the plant than in the soil, by which we can estimate the degree of plant metal uptake (Baker 1981; McGrath and Zhao 2003). When testing this criterion, one should be aware of the mosaic structure of metal pollutants in soil, resulting in different estimates of pollution when different sampling methods are used. In addition, localized hotspots of high metal concentrations are frequently created around perennial hyperaccumulating plant species, as is the case with *T. praecox* (Vogel-Mikuš et al. 2005, 2007; Regvar et al. 2006). It is therefore of extreme importance that the rhizosphere soil (i.e., in contact with the root system) is collected together with plant material to make a realistic estimate of a specific plant/soil concentration ratio (Vogel-Mikuš et al. 2005; Reeves 2006).
3. The shoot to root concentration ratio, frequently termed the *translocation factor* (TF = C_{shoot} /C_{root}), must be invariably higher than 1 (McGrath and Zhao 2003; Baker 1981; Vogel-Mikuš et al. 2005), indicating efficient ability to transport metals from roots to shoots and the existence of tolerance mechanisms to cope with high metal concentrations.

2.1.1 Plant Communities with Metal Hyperaccumulating Plants

Many mineralized areas have a characteristic flora that may be unique to that particular locality or may be specific for all discrete areas of mineralization in that region. It is among such characteristic floras that most, if not all, metal hyperaccumulating plants are to be found (Brooks 1998). The phytosociological communities that comprise these floras have been first described by Ernst (1974). They include:

1. *Serpentine floras* developed on serpentine soils derived from Fe- and Mg- rich ultramafic rocks occurring in many parts of the world that are significantly enriched in Ni, Cr, and Co and deficient in several nutrients, and which are the host to one of the most important groups of hyperaccumulating plants, the so-called *nickel plants* (Brooks 1998), among them being several Brassicaceae (89 taxa), Euphorbiaceae (87 taxa), Asteraceae (44 taxa), Flacourtiaceae (19 taxa), Buxaceae (17 taxa) and Rubiaceae (17 taxa) species. About 90 other species from more than 30 different families belonging to this group are distributed widely throughout the plant kingdom, emphasizing the way in which this property must have evolved independently many times (Brooks 1998; Reeves 2006).

2. *The floras on zinc/lead/copper sulphide mineralization* developed on the so-called *"calamine" soils*, which besides elevated Zn and Pb, usually contain elevated Cd concentrations and occasionally high concentrations of As and/or Cu, belonging to the *Violetum calaminarie westfalicum* association (Ernst 1964; cited after Brooks 1998). Besides *Viola calaminaria,* typical members of zinc/lead floras also include *Minuartia verna* (Caryophyllaceae), *Armeria maritima* (Plumbaginaceae)*, Arabidopsis (Cardaminopsis) halleri, Thlaspi calaminare* and *Thlaspi caerulescens* (Brassicaceae) (Brooks 1998).
3. *Selenium floras* developed on soils derived from various Se-rich rock types, with Se concentrations of >10 mg kg^{-1}, but sometimes exceeding 50 mg kg^{-1} with notable plant genera in which extreme accumulation of Se can be found, including *Astragalus* (Legumuniseae), *Stanleya* (Brassicaceae), *Haplopappus* and *Machaeranthera* (Asteraceae) (Brooks 1998; Reeves 2006).
4. *Copper/cobalt floras of Central Africa* developed on Cu and Co containing soils, such as those of the Shaban Copper Arc in the Democratic Republic of Congo (Brooks 1998; Reeves 2006). Studies of the succession of metallophyte floras of such individualistic nature are of extreme importance in order to determine plant species suitable for successful reestablishment of productive ecosystems in contemporary phytoremedial activities and thus deliberately direct secondary succession on metal contaminated sites in a desired fashion (Wiegleb and Felinks 2001; Regvar et al. 2006).

3 Arbuscular Mycorrhiza in Metal-Contaminated Environments

3.1 Mycorrhizal Status of Plants on Metal-Contaminated Soils

In general, initial colonizers of heavily disturbed and metal-contaminated soils are metal-tolerant plant species, which tend to be nonmycorrhizal or develop low arbuscular mycorrhizal (AM) colonization levels, with important impacts on the increase of soil organic matter content and improvement of the soil microclimate. This tends to be conducive to the establishment of plant species favoring higher AM colonization levels and/or favoring other mycorrhizal types, particularly ericoid- and ectomycorrhizal, and the mycorrhizal succession can therefore be seen as a gradual replacement of nonmycorrhizal by mycorrhizal plant species (Allen 1991; Pawlowska et al. 1996; Leyval and Joner 2001; Regvar et al. 2006).

The reduced arbuscular mycorrhizal fungal (AMF) spore diversity, spore density and AMF infectivity commonly observed in metal-polluted soils was frequently seen as the main factor influencing the observed low mycorrhizal colonization levels (Pawlowska et al. 1996; Leyval and Joner 2001; Regvar et al. 2001). Despite that, several *Glomus* species (e.g., *G. mosseae, G. fasciculatum, G. intraradices, G. aggregatum,* and *G. constrictum*) along with *Scutellospora dipurpurescens*,

Gigaspora sp. and *Entrophospora* sp. were frequently identified on the basis of spore morphology in metal-polluted habitats (Griffioen 1994; Pawlowska et al. 1996, 2000; Regvar et al. 2001).

Studies of the occurrence of mycorrhizal symbiosis in the metal-rich lateritic soils of New Caledonia, where almost 75% of vegetation is endemic, showed that all examined plants were consistently colonized by AMF. The presence of spores of AMF found in their rhizosphere soil indicated the existence of specific mechanisms to control high Fe, Ni, Cr, Co, Mn and Al, and to cope with low nutrient levels in soil, resulting from the coevolution of plants and their fungal symbionts over millions of years. Regardless of the high fungal metal tolerance, however, the lowest AMF spore densities observed in soils with the highest Ni concentrations (Perrier et al. 2006) seem to be a feature frequently found on metal-polluted sites, indicating strong selection pressure(s) on the indigenous AMF populations in metal-enriched soils.

Some plants are clearly more mycorrhizal than others and despite frequently observed seasonal variations a consistent hierarchy is maintained (Peat and Fitter 1993; Fitter and Merryweather 1992; Regvar et al. 2006). Recent studies show that mycorrhizal grasses (e.g., *Agrostis capillaris, Sesleria caerulea, Calamagrostis varia*) that usually colonize Zn-, Cd- and Pb-polluted mining sites (Leyval and Joner 2001; Regvar et al. 2006) are able to maintain relatively constant, though moderate (50–80%), levels of mycorrhizal colonization (Iestwaart et al. 1992; Leyval et al. 1997; Regvar et al. 2006), whereas highly mycorrhizal plant species are mostly found dominating less polluted sites (Pawlowska et al. 1996; Regvar et al. 2006). The functional significance of plant colonization levels are still a matter of debate, highlighting the lack of understanding of the formation of particular AM structures in plant roots (Fitter and Merryweather 1992; Allen 2001; Regvar et al. 2006). Vesicles, frequently hard to distinguish from intraradical spores, are more frequently formed on the most polluted locations and are seen as a part of the mycorrhizal survival strategy on metal-polluted sites (Pawlowska et al. 1996; Turnau et al. 1996; Regvar et al. 2006). The increased proportion of colonized root length at low soil metal concentrations suggests that plants invest increasingly more in AM symbiosis at low soil metal levels (Audet and Charest 2007). However, plant and fungal metal tolerance mechanisms as well as specific edaphic conditions (e.g., soil metal concentrations, metal speciation, soil pH and organic matter contents) should be taken in account when interpreting such results (Leyval et al. 1997; Leyval and Joner 2001; Audet and Charest 2007).

In conclusion, despite the commonly observed low AMF spore diversities and densities in metal-enriched soils, the existing fungal colonizers are presumably the best suited to cope with the existing microclimatic/microedaphic conditions and should therefore be investigated in contemporary conservation practices. The reduced mycorrhizal colonization levels frequently seen within plant communities from metal-contaminated sites seem to arise from both the composition of the plant communities, usually comprised of plant species with low to moderate mycorrhizal colonization levels, and low levels of mycorrhizal colonization of the specimens of particular plant species on the more polluted locations, which are, however, still

within the limits for that plant species, thus maintaining the consistent hierarchy. In addition, intense formation of intraradical fungal spores is frequently found at the most polluted locations. All these characteristics of mycorrhizal colonization may be seen as a plant "mycorrhizal strategy" on highly metal-contaminated environments, contributing significantly to increased plant fitness.

3.2 Functional Significance of Arbuscular Mycorrhizae in Metal-tolerant Plants

Arbuscular mycorrhizal fungi (*Glomeromycota*) (Schüssler et al. 2001) are ubiquitous soil microbes considered essential for the survival and growth of plants in nutrient-deficient soils. They expand the interface between plants and the soil environment and contribute to plant uptake of macronutrients and micronutrients, as well as significantly to stress alleviation (Smith and Read 1997; Allen 1991). In addition, they were shown to protect plants from harmful effects of excess metals, connected mainly to fungal metal tolerance mechanisms (Gildon and Tinker 1983; Dehn and Schüepp 1989; Hetrick et al. 1994; Hildebrand et al. 1999; Chen et al. 2003; Gaur and Adholeya 2004). They are therefore frequently seen as a tolerance mechanism of plants in highly metal-polluted soils (Turnau et al. 1996; Hall 2002; Regvar et al. 2006).

Studies aiming to explain the physiological mechanism(s) involved in metal tolerance of AMF to elevated metals face difficulties in demonstrating their possible role(s) in metal uptake or contribution to the metal tolerance of the host due to their obligate symbiotic character. Numerous effects of AMF on plant metal accumulation may be seen as an array of responses from decreased toward neutral or even increased metal uptake, depending on different plant–AMF combinations and the metals involved (Leyval et al. 1997; Leyval and Joner 2001; Malcova et al. 2003), thus making it difficult to draw any firm conclusions. Despite that, numerous studies have demonstrated that the selection of indigenous metal-tolerant isolates seems to serve the aim of reducing the endogenous concentrations of metals in plants better than nontolerant ones. For example, AMF inoculum from metal-tolerant *Viola calaminaria* was efficient in sequestering metals (e.g., Cd and Zn) in the roots of subterranean clover (Tonin et al. 2001). *Glomus intraradices* Br isolated from the roots of *V. calaminaria* improved maize growth in polluted soil and reduced root and shoot metal concentrations in comparison to a common *G. intraradices* isolate or noncolonized controls (Hildebrandt et al. 1999; Kaldorf et al. 1999), and AMF isolates from As-contaminated soil were proved to confer enhanced arsenate resistance on the grass species *Holcus lanatus* by suppressing As uptake (Gonzales-Chavez et al. 2002). Greater accumulation of Cd, Ti and Ba was observed in fungal structures than in the host plant cells (Turnau et al. 1993), and measurements of the metal-binding capacity of mycorrhizal mycelium showed AMF hyphae have a high metal adsorption capacity (e.g., for Cd), potentially representing a barrier for metal translocation to plant tissues (Joner et al. 2000). Surface adsorption mechanisms

involve ion-exchange, complexation, precipitation and crystallisation of metals on the extra- and intraradical hyphal cell wall components (e.g., chitin, cellulose derivatives and melanin) or extracellular slime, which may reduce the intracellular accumulation of metals and their effects on cytoplasmic processes (Turnau et al. 1993; Galli et al. 1994; Denny and Ridge 1995). Cd-induced transcript levels of a putative Zn transporter gene (*GintZnT1*) of the cation diffusion facilitator family (CDF) were observed in symbiotic mycelia of *Gigaspora margarita,* and of an ABC putative transporter gene (*GintABC1*) of the external mycelia of *Glomus intraradices*, thought to be involved in Cd detoxification in metal-contaminated soils (González-Guerrero et al. 2005, 2006). Mechanisms within fungal cells, however, involve chelation of metal ions by ligands like polyphosphates and metallothioneins, compartmentation within vacuoles, and coping with oxidative stress (Turnau et al. 1993; Kaldorf et al. 1999; Joner et al. 2000; Leyval and Joner 2001; Ouziad et al. 2005; Hildebrandt et al. 2007), and vesicles might potentially serve as storage compartments for heavy metals (Turnau 1998; Weiersbye et al. 1999).

A study on the significance of AM as a function of low/high nutrient availability demonstrated higher biomass production and nitrogen content of mycorrhizal plants under low-nutrient treatment (Cruz et al. 2004). Similar trends seem to apply for uptake of the nonessential elements. The greater volume of the mycorhizosphere, compared to the rhizosphere alone, provides an increased access to soil resources, including macro-, micro-, but also nonessential elements at low soil concentrations. On the other hand, at higher soil metal levels, AMF are expected to reduce soil metal bioavailability since metals are sequestered in extraradical hyphae (Joner et al. 2000), therefore resulting in lower metal uptake in AM than non-AM plants.

In a literature survey, Audet and Charest (2007) correlated metal uptake and relative plant growth parameters of AM plants in heavy metal polluted soils at low and high soil metal concentration intervals. They were able to determine (1) an increased mycorhizospheric metal uptake at low metal concentrations, and (2) a reduced metal uptake via AMF "metal binding" processes at high soil metal levels, hence resulting in enhanced plant tolerance through metal stress avoidance, whereas in the area of transition between the two zones (3) there is kinetic equilibrium that shows no detectable differences between AM and non-AM plants (Fig. 2). Interestingly, far smaller differences are seen on the basis of biomass, with the higher plant biomass of AM plants in the zone of high metal levels being the most prominent (Audet and Charest 2007).

Taken together, the tolerance mechanisms AMF develop at metal-contaminated sites may play a crucial role(s) in mediating metal uptake and translocation to plants (Leyval et al. 1997), with immobilization of metals in the fungal biomass as the main mechanism involved (Zhu et al. 2001; Li and Christie 2000; Gaur and Adholeya 2004). Because of the lower sensitivity of fungal hyphae to metals compared to plant roots (Joner and Leyval 2001), functional symbiosis with metal-tolerant AMF strains may confer improved metal tolerance on plants, while maintaining an adequate supply of nutrients like P and N through active hyphal uptake (Gaur and Adholeya 2004), thus contributing to improved plant fitness in metal-contaminated soils. However, a range of environmental factors including soil metal concentrations and their bioavailability, soil absorption/desorption characteristics, as well as

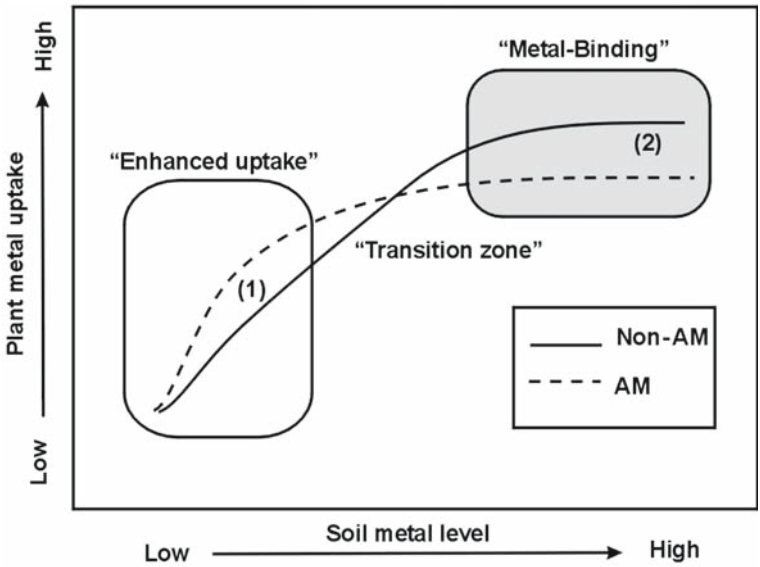

Fig. 2 Conceptual model of plant metal uptake in relation to soil-metal concentration. Designated are zones *Enhanced uptake* and *Metal-Binding* showing greater uptake for AM than non-AM plants at lover soil-metal levels *(1)* and lower uptake for AM than non-AM plant at higher soil-metal levels *(2)* (Reprinted from Audet and Charest 2007, with permission from Elsevier)

endogenous factors (e.g., the fungal properties and inherent heavy metal uptake capacity of plants) may influence the uptake of metals by mycorrhizal plants (Leyval et al. 1997; Pawlowska and Charvat 2004). Considering the compromise between plant growth and metal tolerance, AM plants most likely invest more in a stress-avoidance strategy via metal binding by AMF rather than in metabolically more costly stress-resistance alternatives, such as metal chelation and sequestration (Audet and Charest 2007). This was already indicated by a study on tomato gene expression in metal-polluted soils with induction of plant *Lemt2* (metallothionein) and *LeNramp1* (metal transporter) genes, whose products are putatively involved in metal stress alleviation and that were not induced in the otherwise better growing AM plants. It was suggested that colonization lowered the concentration of heavy metals in plant cells to a level insufficient to induce the expression of these genes (Ouziad et al. 2005; Hildebrandt et al. 2007), which may also apply to more tolerant plant species.

4 AM in Metal Hyperaccumulating Plants

The mycorrhizal association of metal-tolerant plants with indigenous AMF results from a long-term co-evolutionary compromise between the costs and benefits provided to the symbionts (Sanders and Fitter 1992; Whitfield et al. 2004). Many metal

hyperaccumulating plants, however, belong to the plant families Brassicaceae and Caryophyllaceae, that are widely known to possess no or weakly effective associations with AMF (Harley and Harley 1987; De Mars and Boerner 1996), and therefore studies of interactions of metal hyperaccumulating plants with AMF have been generally put aside. Recently, however, mycorrhizal colonization was reported in nickel hyperaccumulating plants belonging to the Asteraceae (Turnau and Mesjasz-Przybylowicz 2003) and *Phyllanthus favieri* (Euphorbiaceae) (Perrier et al. 2006), arsenic hyperaccumulating Pterydophyta (Agely et al. 2005; Liu et al. 2005) and Cd and Zn hyperaccumulating *Thlaspi praecox* (Brassicaceae) (Vogel-Mikuš et al. 2005, 2006).

Studies on the occurrence of mycorrhizal symbiosis in *Phyllanthus favery* from New Caledonia, hyperaccumulating up to 40,000 mg kg^{-1} Ni in leaf dry biomass, showed the plants to be distinctly colonized with AMF. Plant specimens with higher mycorrhizal frequencies had lower Ni concentrations in their leaves compared to non-mycorrhizal individuals. The inhibition of mycorrhizal colonization in *P. faviery* plants at >30,000 mg kg^{-1} Ni in their leaves was attributed to the increased bioavailable Ni in the rhizosphere soil, causing a significant reduction of AMF spore density and AMF infectivity (Perrier et al. 2006). AM symbiosis was also reported in four Ni hyper accumulating representatives of the Asteraceae: *Senecio anomalochrous*, *S. coronatus*, *Berkheya codii* and *B. zeyherii* from South Africa. The degree of mycorrhizal colonization was high (F > 80%) in most collected plant specimens, with well-developed intraradical colonization of *Arum* type (Turnau and Mesjasz-Przybylowicz 2003). In a greenhouse experiment with Ni hyperaccumulating *Berkheya codii,* higher shoot biomass and higher Ni uptake was observed in the plants inoculated with native AMF, when compared to noninoculated plants or plants inoculated with nontolerant *Glomus intraradices* (BEG). The poor arbuscular development observed after inoculation with nontolerant *G. intraradices* was attributed to the deleterious effect of Ni on the fungus and/or to possible plant restriction(s) towards a nonbeneficial fungus (Turnau and Mesjasz-Przybylowicz 2003). Similarly, inoculation of the As hyperaccumulator *Pteris vittata* with indigenous AMF from an As-contaminated site resulted in increased frond dry mass and in increased As uptake (Agely et al. 2005). The inoculation of *P. vittata* with *Glomus mossae* BEG 167, not originating from As- contaminated soils on the other hand, increased frond dry matter but significantly decreased frond As uptake (Liu et al. 2005).

More data on mycorrhizal colonization of hyperaccumulating plants are expected in coming years because of its importance for plant fitness in phytoremediation. It has become clear that an extensive portion of the root system should be analyzed in order to find the low levels of colonization (e.g., up to 4%) of the root segments that may otherwise be easily overlooked (Regvar et al. 2006). Using the method of Trouvelot et al. (1986), we were able to detect mycorrhizal colonization as well as colonization, with dark septate endophytes in plants from intermittent aquatic habitats even though the root system of every plant was not colonized (Šraj-Kržič et al. 2006). Therefore, several environmental factors affecting mycorrhizal colonization (e.g., metal levels, soil erosion, plant succession, organic matter

content, mineral contents, etc.) should be objectively evaluated in the search for mycorrhizal colonization of hyperaccumulating plants. Because of the lack of experimental data on AM colonization and its function(s) in metal hyperaccumulating plants, the last part of this chapter will be focused on the genus *Thlaspi* with *T. caerulescens* being a model organism for studies of metal hyperaccumulation and *T. praecox* a Cd/Zn hyperaccumulator forming distinct symbiosis with AMF.

4.1 Genus Thlaspi

4.1.1 AM Status of Genus *Thlaspi*

Screening of AMF colonization in different species from the genus *Thlaspi* revealed that the species growing in an active meadow community (e.g., *T. praecox*, *T. sylvestre* = *T. caerulescens* (Banjščice) and *T. montanum*) were distinctly colonized by AMF, while lower colonization levels with only fungal hyphae were observed in the roots of the species collected at metal-polluted sites (e.g., *T. caerulescens, T. goesingense, T. calaminare* and *T. cepaeifolium*) (Regvar et al. 2003). Among the reasons for the observed differences may be the high energy demands the plants have to expend on metal tolerance mechanisms in metal-polluted environments (Baker 1987) instead of investing in AM symbiosis (Regvar et al. 2006). Different morphology of AMF structures is another feature of the AM symbiosis observed in *T. praecox* roots from polluted sites with typical *Paris* type morphology, characterized by typical arbuscule development on coils (Fig. 3a), that was attributed to the adaptation of one or possibly both symbionts to stressful conditions. In contrast, a more *Arum* type morphology (Fig. 3b) was seen at the nonpolluted sites (Vogel-Mikuš et al. 2005; Regvar et al. 2003). Identification of AMF from

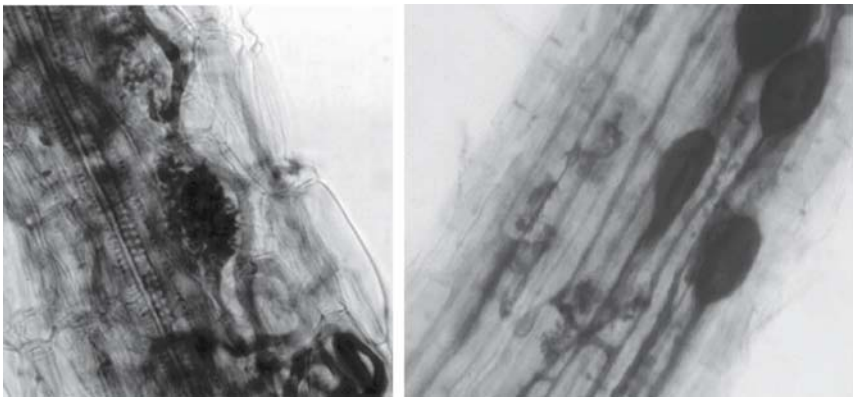

Fig. 3 **a** *Paris* and **b** *Arum* type of *Thlaspi praecox* AM colonization

nonpolluted sites using modern molecular techniques revealed the selected species from the genus *Thlaspi* were colonized by common AMF belonging to the *Glomus intraradices* clade. However, none of the sequences obtained matched any other deposited in databanks, indicating the existence of a species continuum in the *G. intraradices* clade (Regvar et al. 2003). Further studies will be needed to identify AMF colonizing *T. praecox* at metal-polluted sites in order to shed more light on the role of AMF taxonomy in AM symbiosis. Nevertheless, as it was recently demonstrated that different AM morphology may be observed in the co-occurring plant species harboring similar AMF species (Ahulu et al. 2006), factors other than AMF species may be decisive in determining the type of AM morphology in hyperaccumulating plants.

4.1.2 Physiology of Metal Hyperaccumulation in *Thlaspi* Species

Metal hyperaccumulation of *Thlaspi* species is closely connected to physiological and biochemical adaptations of the root metal uptake, metal transport from the roots to the shoots, and metal sequestration and detoxification in shoots or leaves (Baker 1987; Ernst et al. 1992; Hall, 2002); these mechanisms have already been exhaustively reviewed (Vogel-Mikuš and Regvar 2006). The roots of the Zn/Cd hyperaccumulator *T. caerulescens* preferentially spread towards areas with higher Zn or Cd concentrations in soil (Whiting et al. 2000; Haines, 2002). In addition, overexpression of Zn transporters in the roots of *T. caerulescens* was observed when compared to nonaccumulating *T. arvense* (Lasat et al. 1996). In *T. caerulescens,* up to five times more Zn was observed in xylem sap compared to *T. arvense*, since Zn is not sequestered in the root vacuoles, but rather it is successfully loaded from the cytoplasm to the xylem (Lasat et al. 1998). Within the xylem, the metals are transported as stable complexes bound either to free His (as His-Ni and His-Zn complexes), to nicotianamine, organic acids (Krämer et al. 1996; Küper et al. 2004; Pilon-Smits 2005) or S-ligands (Cd) (Küper et al. 2004). Within the leaves, metals are usually accumulated along the path of the transpiration stream, in epidermal and vascular tissues and to a lesser extent in palisade and spongy mesophyll, indicating metal accumulation away from photosynthetically active tissues (Seregin and Ivanov 2001; Bhatia et al. 2004; Cosio et al. 2005; Wójcik et al. 2005). Zn was proven to be localized in large vacuolated epidermal cells (Frey et al. 2000). At the cellular level, metals are usually sequestered in cell vacuoles bound to different ligands (Salt and Krämer 2000).

Compared to plant species that are unable to tolerate excessive soil metal concentrations, metal hyperaccumulating plants also had to adapt their reproductive period in order to maintain their fitness. During development of seeds, metals are transported from leaves by the phloem stream, so metal uptake depends basically on the phloem loading within leaves (Marschner 1995). In metal-tolerant plants, efficient metal sequestration mechanisms maintain low free metal concentrations in the symplast, thus limiting metal phloem loading and transport from the leaves to the seeds (Lasat et al. 1998; Wójcik et al. 2005). Therefore, seeds usually contain

lower metal concentrations than any other plant part, thus enabling the survival of the embryo in metal-polluted environments (Ernst et al. 1992; Psaras and Manetas 2001). Frequently, the seed coat and/or endosperm represent(s) another barrier preventing metal accumulation in embryonic tissues (Ernst et al. 1992; Mesjazs-Przybylowicz et al. 2001; Bhatia et al. 2003). However, few data on the accumulation and distribution of metals in seeds of metal hyper accumulating plants are available. In the Ni hyperaccumulator *Stackhousia tryionii,* Ni is mainly localized in the pericarp, with an order of magnitude lower concentration found in the endosperm and embryonic tissues (Bhatia et al. 2003). Within the seeds of *T. pindicum,* the highest Ni concentrations were observed in the seed-coat micropylar region, while in the embryo Ni was mainly found in the epidermis of cotyledons (Psaras and Manetas 2001). In the seeds of the Zn/Cd hyperaccumulator *T. praecox,* an order of magnitude higher Cd than Zn concentrations were found, with the highest concentrations of both metals observed in embryonic tissues, mainly in the epidermis of cotyledons. Metal partitioning away from the ancestral photosynthetically active tissues and the radicle of the developing seedlings may represent a tolerance strategy enabling reproductive success at metal polluted sites. Nevertheless, seed biomass and viability decreased with increased seed Cd concentrations (Vogel-Mikuš et al. 2007).

4.1.3 Functional Significance of AM in *T. praecox*

Considerable scepticism, however, arose as to the meaningfulness of fungal colonization of *Thlaspi* species after all initial inoculation attempts failed (Regvar et al. 2003). Only later, however, was it realised that plants need to be vernalized in order to form mycorrhizae under laboratory conditions (Vogel-Mikuš et al. 2006). AM symbiosis in *T. praecox* was established with indigenous fungal symbionts from the metal-polluted site in order to increase the likelihood of meeting of compatible symbiont(s). Colonization was only observed during the flowering period and no positive effect of mycorrhizal colonization on plant biomass was found. Nevertheless, the contents of mineral nutrients e.g., phosphorus, significantly increased in inoculated plants, indicating functional exchange of nutrients and presumably carbohydrates between the partners (Vogel-Mikuš et al. 2006) in line with Smith (2000). A *Paris*-type of AMF morphology with limited arbuscular development formed usually on coils was observed under controlled conditions, indicating hyphae and coils may also play an important role in the exchange of nutrients (Vogel-Mikuš et al. 2006). Colonized plants showed lower Cd concentrations in roots and shoots, and lower root Zn concentrations. Zn accumulation in shoots of AM plants increased at lower soil Zn concentrations, whereas at higher Zn concentrations no differences were observed compared to noncolonized plants. In addition, the uptake strategy of Pb into *T. praecox* roots was changed in the presence of AMF, with higher root Pb concentrations in AM plants at lower soil Pb contents and lower Pb concentrations in AM plants at higher soil Pb contents (Vogel-Mikuš et al. 2006), in line with the conceptual model proposed by Audet and Charest (2007). Besides an improved

mineral nutrition, the results indicate changes in elemental uptake strategies of AM. *T. praecox*, reflecting the protective role of AMF in metal-polluted soils (Gildon and Tinker 1983; Dehn and Schüepp 1989; Hetrick et al. 1994; Hildebrandt et al. 1999; Chen et al. 2003; Vogel-Mikuš et al. 2005, 2006; Audet and Charest 2007).

Improved nutrient uptake is of extreme importance during the reproductive period, resulting in the production of high quality seed reserves (Koide and Lu 1992; Allsopp and Stock 1992; Jacobsen et al. 2002) with obvious consequences for early plant establishment and survival of specimens in nutrient-limited environments typical of metal-polluted sites (Marschner 1995, 1998; Adriano 2001; Ernst 2005). Seeds of *T. praecox* from a metal-polluted site showed significantly decreased biomass, germination potential and viability, accompanied by increased seed dormancy when compared to seeds collected at a nonpolluted site. In addition, the seeds showed severe Cd hyperaccumulation with up to 1,350 μg g^{-1} Cd, with the epidermis of cotyledons as the primary storage tissue showing concentrations of 1,800 μg g^{-1} Cd dry weight (Vogel-Mikuš et al. 2007).

The outcome of the interaction between a hyperaccumulating plant and its potential symbiont(s) seems to result from a sensitive equilibrium between the costs and benefits of mycorrhizal colonization for the plant against the costs of the metal tolerance mechanisms hyperaccumulating plants need to apply at metal-polluted environments in order to ensure sufficient fitness for the species. Generally, higher mycorrhizal colonization levels of hyperaccumulating plants are seen at less polluted environments, particularly due to the higher diversity and infectivity of AMF species. In addition, direct or indirect stressful environmental conditions presumably contribute to the differences in the AM morphology seen in *T. praecox* at polluted compared to nonpolluted sites. Hyperaccumulating plants of the Brassicaceae favor mycorrhizal colonization, particularly during the reproductive period, in order to ensure improved mineral nutrition accompanied by decreased metal levels during the periods of flowering and seeding, resulting in more viable seed banks and better conditions for germination and offspring survival.

4.2 The Need to Study Mycorrhizae in Metal Hyperaccumulating Plants

From an ecotechnological viewpoint, succession research may either be viewed as an accompanying element of revegetation experiments to reestablish productive ecosystems, or as an instrument to deliberately direct succession in a desired fashion. Any decision making in contemporary conservation management practice should be based on a solid analysis of the site and related to justified conservation aims. However, there is a lack of knowledge on the course of the succession of the vegetation of post-mining landscapes that are floristically complex and contain several exclusive species of highly individualistic nature and are thus not easily compared to those of undisturbed areas (Wiegleb and Felinks 2001). Metal hyperaccumulating plants in particular represent an unusual and valuable biological resource with great

potential for use in a variety of strategies for soil phytoremediation. Due to their high metal tolerance, they are unique members contributing in an individualistic manner to the vegetation composition at highly metal-contaminated environments and affecting either natural or anthropogenically assisted secondary succession under such highly stressful environments. AM associations are also an important feature of natural and managed ecosystems due to their nutritional and nonnutritional benefits to their symbiotic partners in altering plant diversity and productivity, because they can act as biofertilizers, bioprotectants or biodegraders, and the fact that AMF occur in contaminated soils makes "adapted" indigenous inocula a promising tool for soil phytoremediation efforts (Van der Heijden et al. 1998; Khan 2005; Göhre and Paszkowski 2006). The potential role of AM symbiosis in phytoremediation was already exhaustively reviewed elsewhere (Vogel-Mikuš and Regvar 2006) and should be particularly considered for the facilitation of the succession and improvement of plant tolerance and fitness in highly polluted soils. The product of biomass and metal concentration is the single most important quantity defining the suitability of metallophytes for phytoextraction (Bennett et al. 1998). Among metal hyperaccumulating plants, the perennial *T. goesingense* and *T. montanum*, the Ni hyper accumulator *Berkheya codii* and the As hyperaccumulator *Pteris vittata* are fairly productive and therefore appear to be suitable for that purpose. Inoculation of Ni hyperaccumulating *Berkheya codii* or As hyperaccumulating *Pteris vittata* with indigenous AMF also seems to be promising because it increases both plant biomass and metal uptake (Turnau and Mesjasz-Przybylowicz 2003; Agely et al. 2005). However, further research is needed to fully explore the potential of AM symbioses in the majority of metal hyperaccumulating plants before significant progress in AMF hyperaccumulating plant phytoremediation technologies can be made.

References

Adriano DC (2001) Trace elements in terrestrial environments, biochemistry, bioavailability and risk of metals. Springer, Heidelberg

Agely AA, Sylvia DM, Ma LQ (2005) Mycorrhizae increase arsenic uptake by the hyperaccumulator Chinese brake fern (*Pteris vittata* L.). J Environ Qual 34:2181–2186

Ahulu EA, Gollotte A, Gianinazzi-Pearson V, Nonaka M (2006) Cooccurring plants forming distinct arbuscular mycorrhizal morphologies harbor similar AM fungal species. Mycorrhiza 17:37–49

Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, Cambridge

Allen MF (2001) Modelling arbuscular mycorrhizal infection: is % infection an appropriate variable? Mycorrhiza 10: 255–258

Allsopp N, Stock WD (1992) Mycorrhizas, seed size and seedling establishment in a low nutrient environment. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhiza in ecosystems. CABI, Wallingford, UK, pp 59–64

Audet P, Charest C (2007) Dynamics of arbuscular mycorrhizal symbiosis in heavy metal phytoremediation: metaanalytical and conceptual perspectives. Environ Pollut 147:609–614

Baker AJM (1981) Accumulators and excluders - strategies in the response of plants to heavy metals. J Plant Nutr 3:643–654

Baker AJM (1987) Metal tolerance. New Phytol 106:93–111

Baker AJM, Brooks RR (1989) Terrestrial higher plants which hyperaccumulate metallic elements- a review of their distribution, ecology and phytochemistry. Biorecovery 1:81–126

Bennett FA, Tyler EK, Brooks RR, Gregg PEH, Stewart RB (1998) Fertilisation of hyperaccumulators to enhance their potential for phytoremediation and phytomining. In: Brooks RR (ed) Plants that hyperaccumulate heavy metals. CABI New York, pp 249–259

Bhatia NP, Orlic I, Siegele R, Ashwath N, Baker AJM, Walsh KB (2003) Elemental mapping using PIXE shows the main pathway of nickel movement is principally symplastic within the fruit of the hyperaccumulator *Stackhousia tryonii*. New Phytol 160:479–488

Bhatia NP, Orlic I, Siegele R, Ashwath N, Baker AJM, Walsh KB (2004) Quantitative cellular localisation of nickel in leaves and stems of the hyperaccumulator plant *Stackhousia tryonii* Bailey using nuclear-microprobe (Micro-PIXE) and energy dispersive X-ray microanalysis (EDXMA) techniques. Funct Plant Biol 31:1–14

Brooks RR (1998) Plants that hyperaccumulate heavy metals. CABI, New York

Brooks RR, Lee J, Reeves RD, Jaffré T (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J Geochem Explor 7:49–57

Chen BD, Tao HQ, Christie P, Wong MH (2003). The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc. Chemosphere 50:839–846

Cosio C, DeSantis L, Frey B, Diallo S, Keller C (2005). Distribution of cadmium in leaves of *Thlaspi caerulescens*. J Exper Bot 56:765–775

Cruz C, Green JJ, Watson CA, Wilson F, Martins-Loução MA (2004) Functional aspects of root architecture and mycorrhizal inoculation with respect to nutrient uptake capacity. Mycorrhiza 14:177–184

De Mars BG, Boerner REJ (1996) Vesicular arbuscular mycorrhizal development in the Brassicaceae in relation to plant life span. Flora 191:179–189

Dehn B, Schüepp H (1989) Influence of VA mycorrhizae on the uptake and distribution of heavy metals in plants. Agriculture. Ecosyst Environ 29:79–83

Denny HJ, Ridge I (1995). Fungal slime and its role in the mycorrhizal amelioration of zinc toxicity to higher plants. New Phytol 130: 251–257

Ernst W (1964) Ökologisch-soziologische Untersuchungen in den Schwermetall-gesellschaften Mitteleuropas unter Einschluß der Alpen. Abhhandlungen des Landesmuseums für Naturkunde 27: 1–54

Ernst WHO (1974) Schwermetallvegetation der Erde. Fischer, Stuttgart

Ernst WHO (2005) Phytoextraction of mine wastes – options and impossibilities. Chem Erde 65:29–42

Ernst WHO, Verkleij JAC, Schat H (1992). Metal tolerance in plants. Acta Bot Neerland 41:229–248

Fitter AH, Merryweather JW (1992) Why are some plants more mycorrhizal than others? An ecological enquiry. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CABI, Wallington, pp 26–36

Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 23:675–687

Galli U, Schuepp H, Brunold C (1994) Heavy metal binding by mycorrhizal fungi. Physiol Plant 92:364–368

Gaur G, Adholeya A, (2004). Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Curr Sci 86:528–534

Gildon A, Tinker PB (1983) Interactions of vesicular-arbuscular mycorrhizal infection and heavy metals in plants, I. The effects of heavy metals on the development of vesicular-arbuscular mycorrhizas. New Phytol 95:247–261

Glass DJ (2000) Economic potential of phytoremediation. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals. Using plants to clean up the environment. Wiley, New York, pp 15–31

Göhre V, Paszkowski U (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 223:1115–1122

Gonzalez-Chavez C, Harris PJ, Dodd J, Meharg AA (2002) Arbuscular mycorrhizal fungi confer enhanced arsenate resistance on *Holcus lanatus*. New Phytol 155:163–171

González-Guerrero M, Azcon-Aguilar C, Mooney M, Valderas A, MacDiarmid CW, Eide DJ, Ferrol N (2005) Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Fungal Genet Biol 42:130–140

González-Guerrero M, Azcon-Aguilar C, Ferrol N (2006) GintABC1 and GintMT1 are involved in Cu and Cd homeostasis in *Glomus intraradices*. In: Abstracts of the 5th International Conference of Mycorrhiza, Granada, Spain

Griffioen WAJ (1994) Characterization of a heavy metal-tolerant endomycorrhizal fungus from the surroundings of a zinc refinery. Mycorrhiza 4:197–200

Haines BJ (2002) Zincophilic root foraging in *Thlaspi caerulescens*. New Phytol 155:363–372

Hall J.L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. J Exper Bot 366: 1–11

Harley JL, Harley EL (1987) A check-list of mycorrhiza in the British flora. New Phytol 105[suppl]:1–102

Hetrick BAD, Wilson GWT, Figge DAH (1994) The influence of mycorrhizal symbiosis and fertilizer amendments on establishment of vegetation in heavy metal mine spoils. Environ Pollut 86:171–179

Hildebrandt U, Kaldorf M, Bothe H (1999). The zinc violet and its colonization by arbuscular mycorrhizal fungi. J Plant Physiol 154:709–717

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146

Iestwaart JH, Griffioen WAJ, Ernst WHO (1992) Seasonality of VAM infection in three populations of *Agrostis capillaris* (Graminae) on soil with or without heavy metal enrichment. Plant Soil 139:67–73

Jacobsen I, Smith SE, Smith FA (2002) Function and diversity of arbuscular mycorrhizae in carbon and mineral nutrition. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 75–92

Joner E, Briones R, Leyval C. (2000). Metal-binding capacity of arbuscular mycorrhizal mycelium. Plant Soil 226:227–234

Kaldorf M, Kuhn AJ, Schroder WH, Hildebrandt U, Bothe H. (1999). Selective element deposits in maize colonized by a heavy metal tolerance conferring arbuscular mycorrhizal fungus. J Plant Physiol 154:718–728

Khan A G (2005) Role of soil microbes in the rhizosphere of plants growing on trace metal contaminates soils in phytoremediation. J Trace Elements Med Biol 18:355–364

Koide R, Lu X (1992) Mycorrhizal infection of wild oats, Parental effects on offspring nutrient dynamics, growth and reproduction. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhiza in ecosystems. CABI, New York, pp 55–58

Krämer U, Cotter-Howells JD, Charnock JM, Baker AJM, Smith JAC (1996) Free histidine as a metal chelator in plants that accumulate nickel. Nature 378:635–638

Lasat MM, Baker AJM, Kochian LV (1996) Physiological characterisation of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. Plant Physiol 112:1715–1722

Lasat MM, Baker AJM, Kochian LV (1998) Altered Zn compartmentation in root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. Plant Physiol 118:875–883

Leyval C, Joner EJ (2001). Bioavailability of heavy metals in the mycorrhizosphere. In Gobran RG, Wenzel WW, Lombi E (eds) Trace metals in the rhizosphere. CRC Press, Fla., USA, pp 165–185

Leyval C, Turnau K, Haselwandter K (1997) Effect of heavy metal pollution on mycorrhizal colonization and function, physiological, ecological and applied aspects. Mycorrhiza 7:139–153

Li XL, Christie P (2000) Changes in soil solution Zn and pH and uptake of Zn by arbuscular mycorrhizal red clover in Zn-contaminated soil. Chemosphere 42:201–207

Liu Y, Zhu YG, Chen BD, Christie P, Li XL (2005) Influence of the arbuscular mycorrhizal fungus *Glomus mosseae* on uptake of arsenate by the As hyperaccumulator fern *Pteris vittata* L. Mycorrhiza 15:187–192

Malcova R, Vosátka M, Gryndler M (2003) Effects of inoculation with *Glomus intraradices* in lead uptake by *Zea mays* L. and *Agrostis capillaris* L. Appl Soil Ecol 23:55–67

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, London

Marschner H (1998) Role of root growth, arbuscular mycorrhizal, and root exudates for the efficiency in nutrient acquisition. Field Crops Res 56:203–207

McGrath SP, Zhao FJ (2003) Phytoextraction of metals and metalloids from contaminated soils. Curr Opin Biotechnol 14:277–282

Mesjazs-Przybylowicz J, Grodzinska K, Przybylowicz WJ, Godzik B, Szarek-Lukaszewska G (2001) Nuclear microprobe studies of elemental distribution in seeds of *Biscutella laevigata* L. from zinc wastes in Olkusz, Poland. Nucl Instrum Method B 181:634–639

Naidu R, Gupta VVSR, Rogers S, Kookana RS, Bolan NS, Adriano DC (2003) Bioavailability, toxicity and risk relationships in ecosystems. Science Publishers, Enfield

Ouziad F, Hildebrandt U, Schmelzer E, Bothe H (2005) Differential gene expressions in arbuscular mycorrhizal-colonized tomato grown under heavy metal stress. J Plant Physiol 162:634–649

Pawlowska TE, Charvat I (2004) Heavy metal stress and developmental patterns in arbuscular mycorrhizal fungi. Appl Environ Microbiol 70:6643–6649

Pawlowska TE, Blaszkowski J, Rühling Å (1996) The mycorrhizal status of plants colonising a calamine spoil mound in southern Poland. Mycorrhiza 6:499–505

Pawlowska TE, Chaney RL, Chin M, Charavat I (2000) Effects of metal phytoextraction practices on the indigenous community of arbuscular mycorrhizal fungi at a metal-contaminated landfill. Appl Environ Microbiol 66:2526–2530

Peat HJ, Fitter AH (1993) The distribution of arbuscular mycorrhizas in the British Flora. New Phytol 125:843–854

Perrier N, Amir H, Colin F (2006) Occurrence of mycorrhizal symbioses in the metal-rich lateritic soils of the Koniambo Massif, New Caledonia. Mycorrhiza 16:449–458

Pilon-Smits E (2005) Phytoremediation. Annu Rev Plant Biol 56:15–39

Psaras GK, Manetas Y (2001) Nickel localization in seeds of the metal hyperaccumulator *Thlaspi pindicum* Hausskn. Ann Bot 88:513–516

Reeves RD (1992) Hyperaccumulation of nickel by serpentine plants. In: Proctor J, Baker AJM, Reeves RD (eds) The vegetation of ultramafic (serpentine) soils. Intercept, Andover, UK, pp 253–277

Reeves RD (2006). Hyperaccumulation of trace elements by plants. In Morel JL, Echevarria G, Goncharova N (eds.) Phytoremediation of metal contaminates soils. Nato Science Series: IV: Earth and environmental sciences. Springer, Heidelberg, pp 25–52

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Raskin I, Ensley BD (eds) Phytoremediation of toxic metals: using plants to clean up the environment. Wiley, New York

Regvar M, Groznik N, Goljevšček N, Gogala N (2001). Diversity of arbuscular mycorrhizal fungi at various differentially managed ecosystems in Slovenia. Acta Biol Sloven 44:27–34

Regvar M, Vogel K, Irgel N, Wraber T, Hildebrandt U, Wilde P, Bothe H (2003) Colonization of pennycresses (*Thlaspi sp*.) of the Brassicaceae by arbuscular mycorrhizal fungi. J Plant Physiol 160:615–626

Regvar M, Vogel-Mikuš K, Kugonič N, Turk B, Batič F (2006) Vegetational and mycorrhizal successions at a metal polluted site: Indications for the direction of phytostabilisation? Environ Pollut 144:976–984

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13:468–474

Salt DE, Krämer U (2000) Mechanisms of metal hyperaccumulation in plants. In Raskin I, Ensley BD (eds) Phytoremediation of toxic metals: using plants to clean up the environment. Wiley, New York, pp 193–229

Sanders IR, Fitter AH (1992) The ecology and functioning of vesicular-arbuscular mycorrhizas in coexisting grassland species. II. Nutrient uptake and growth of vesicular arbuscular mycorrhizal plants in a semi-natural grassland. New Phytol 120:525–533

Schüssler A, Schwarzott D, Walker C (2001) A new phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421

Seregin IV, Ivanov VB (2001) Physiological aspects of cadmium and lead toxic effects on higher plants. Russ J Plant Physiol 48:523–544

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Smith FA (2000) Measuring the influence of mycorrhizas. New Phytol 148:4–6

Šraj-Kržič N, Pongrac P, Klemenc M, Kldnik A, Regvar M, Gaberščik A (2006) Mycorrhizal colonization in plants from intermittent aquatic habitats. Aquat Bot, in press

Tonin C, Vandenkoornhuyse P, Joner EJ, Straczek J, Leyval C (2001). Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of *Viola calaminaria* and effect of these fungi on heavy metal uptake by clover. Mycorrhiza 10:161–168

Trouvelot A, Kough JL, Gianinazzi-Pearson V (1986) Measure du taux de mycorhization VA d'un systeme radiculare. Recherche de methods d'estimation ayant une signification functionnelle. In: Gianinazzi-Pearson V, Gianinazzi S (eds) Physiological and genetic aspects of mycorrhizae. INRA, Paris, pp 217–221

Turnau K (1998) Heavy metal content and localisation in mycorrhizal *Euphorbia cyparissias* from zinc wastes in southern Polland. Acta Soc Bot Pol 67: 105–113

Turnau K, Mesjasz-Przybylowicz J (2003) Arbuscular mycorrhizal of *Berkheya codii* and other Ni-hyperaccumulating members of Asteraceae from ultramafic soils in South Africa. Mycorrhiza 13:185–190

Turnau K, Kottke I, Oberwinkler F. (1993). Element localization in mycorrhizal roots of *Pteridium aquilinum* (L.) Kuhn collected from experimental plots with cadmium dust. New Phytol 123:313–324

Turnau K, Miszalski Z, Trouvelot A, Bonfante P, Gianinazzi S (1996). *Oxalis acetosella* as a Monitoring Plant on Highly Polluted Soils. In Azcon-Aguilar C, Barea JM (eds) Mycorrhizas in integrated systems, from genes to plant development. Proceedings of the fourth European symposium on mycorrhizas. (COST Edition). European Commission, Brussels, Luxemburg, pp 483–486

Van der Heijden MAG, Klironomus JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998). Mycorrhizal fungal diversity determines plant biodiversity and productivity. Nature 369:69–72

Vogel-Mikuš K, Regvar M (2006) Arbuscular mycorrhiza as a tolerance strategy in metal contaminated soils – prospects in phytoremediation. In: Rhodes D (ed) New Topics in Environmental Research. Nova Science, Hauppauge, N.Y., pp 37–56

Vogel-Mikuš K, Drobne D, Regvar M (2005) Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonization of pennycress *Thlaspi praecox* Wulf. Brassicaceae from the vicinity of a lead mine and smelter in Slovenia. Environ Pollut 133:232–242

Vogel-Mikuš K, Pongrac P, Kump P, Nečemer M, Regvar M (2006) Colonization of a Zn, Cd and Pb hyperaccumulator *Thlaspi praecox* Wulfen with indigenous arbuscular mycorrhizal fungal mixture induces changes in heavy metal and nutrient uptake. Environ Pollut 139:362–371

Vogel-Mikuš K, Pongrac P, Kump P, Nečemer M, Simčič J, Pelicon J, Budnar M, Povh B, Regvar M (2007). Localisation and quantification of elements within seeds of Cd/Zn hyperaccumulator *Thlaspi praecox* by micro-PIXE. Environ Pollut 147:50–59

Weiersbye IM, Straker CJ, Przybylowicz WJ (1999) Micro-PIXE mapping of elemental distribution in arbuscular mycorrhizal roots of the grass, *Cynodon dactylon*, from gold and uranium mine tailings. Nucl Instrum Method B 158:335–343

Whitfield L, Richards AJ, Rimmer DL (2004) Relationships between soil heavy metal concentration and mycorrhizal colonization in *Thymus polytrichus* in northern England. Mycorrhiza 14:55–62

Whiting SN, Leake JR, McGrath SP, Baker AJM (2000) Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. New Phytol 145:199–210

Wiegleb G, Felinks B (2001). Primary succession in post-mining landscapes of Lower Lusatia – chance or necessity? Ecol Eng 17:199–217

Wójcik M, Vangronsveld J, Haen JD, Tukiendorf A (2005) Cadmium tolerance in *Thlaspi caerulescens* II. Localization of cadmium in *Thlaspi caerulescens*. Environ Exper Bot 53:163–171

Zhu YG, Christie P, Laidlaw AS (2001) Uptake of Zn by arbuscular mycorrhizal white clover from Zn-contaminated soil. Chemosphere 42:193–199

Mycorrhizal Fungi and Other Root Endophytes as Biocontrol Agents Against Root Pathogens

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1 Introduction

In nature, production of disease-free plants with enhanced yield and compounds of therapeutic value can be mediated through rhizospheric microorganisms. There are increasing environmental concerns over the widespread use of biocontrol measures in general, and alternatively, more sustainable methods of disease control are now being sought. Plant diseases caused by root pathogens need to be controlled in order to maintain the quality and abundance of food, feed and fiber, the prime necessities of life. Different approaches are used for prevention and control of these root pathogens. Among these alternatives are those referred to as biological control; the most obvious and apparently biological control is a potent means of reducing the damage caused by plant pathogens. The potential agents for biocontrol activity are rhizosphere-competent fungi and bacteria which, in addition to their antagonistic activity, are capable of inducing growth responses by either controlling minor pathogens or by producing growth-stimulating factors.

A variety of biological controls are available for use, but further development and effective adoption requires a greater understanding of the complex interactions among plants, people, and the environment. This article emphasizes: (1) information about mycorrhiza and root endophytes, (2) various definitions and key mechanisms of biocontrol, and (3) the relationships between microbial diversity and biological control.

2 Endophytes

2.1 Definition of an Endophyte

The term endophyte was introduced by De Bary (1866) and was initially applied to any organism found within a plant. Petrini (1991) used the term endophyte to mean all organisms inhabiting plant organs that at some time in their life can colonize

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internal plant tissues without causing apparent harm to the host. This has been the most widely used definition of endophytes and also includes the organisms that have a more or less lengthy epiphytic phase and also latent pathogens (Schulz et al. 1998; Petrini 1991; Ghimire and Hyde 2004).

Studies on the endophyte composition in different hosts have identified organisms with varying roles within their hosts. By analyzing the different levels of endophytic association, Wilson (1995) stated that "endophytes are fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but causes no symptoms of the disease." The same organism may also be described as a saprobe or pathogen at other times (Boddy and Griffith 1989).

2.2 Role of Endophytes

Endophytes previously defined as mutualists are closely related to virulent pathogens but have limited pathogenicity, and have probably evolved directly from plant pathogenic fungi (Carroll 1988). The mutualistic symbiosis includes the lack of destruction of most cells or tissues, nutrient and chemical cycling between the fungus and hosts, enhanced longevity and photosynthetic capacity of cell and tissue under the influence of infection, enhanced survival of fungus, and a tendency towards greater host specificity than is seen in necrotrophic infections (Lewis 1973; Ghimire and Hyde 2004).

In the grasses and other plant hosts, endophytes have also been shown to enhance plant growth, reduce infection by nematodes, increase stress tolerance and increase nitrogen uptake in nitrogen deficit soils (Bacon 1993; Bultman and Murphy 2000; Clay 1987, 1990; Gasoni and Stegman De Gurfinkel 1997; Kimmons 1990; Varma et al. 1999; Jordaan et al. 2006). Several reviews are available on secondary metabolite production by endophytes (Miller 1986; Clay 1991; Tejesvi et al. 2006). Endophytes in culture can produce biologically active compounds (Brunner and Petrini 1992) including several alkaloids, paxilline, lolitrems and tertraenone steroids (Dahlman et al. 1991), antibiotics (Fisher et al. 1984a, 1984b) and plant growth promoting factors (Petrini et al. 1992). Endophytes are increasingly being identified as a group of organisms capable of providing a source of secondary metabolites for the use in biotechnology and agriculture (Bills and Polishook 1992).

2.3 Modes of Endophytic Colonization

The colonization of plant tissues by endophytes, plant pathogens and mycorrhizae involves several steps involving host recognition, spore germination, penetration of the epidermis and tissue colonization (Petrini 1991, 1996). The inoculum source of fungal endophytes is widely considered to be the airborne spores, and also by seed transmission and transmission of propagules by insect vectors (Petrini 1991). In

terms of mechanical and enzymatic elements of penetration by endophytic fungi, it can be assumed that endophytes adopt the same strategy for penetration of host tissue as pathogens (Petrini et al. 1992). Fungi can invade plant tissues by direct cuticular penetration, via appressoria formed on the cuticle, after which penetration occurs through the cuticle and epidermal cell wall, or via natural openings like stomata (O'Donnell and Dickinson 1980; Kulik 1988). Following the penetration, the infection may be intercellular or intracellular and may be limited to one cell or in a limited area around the penetration site. Limited cytological works on nonclavicipitaceous endophytes have shown that the infection of these endophytes in host plants may be inter- or intracellular and often localized in single cells (Stone 1988; Suske and Acker 1989; Ghimire and Hyde 2004).

3 Mycorrhizal Fungi

The roots of most plant species live associated with certain soil fungi. This symbiotic establishment is known as mycorrhiza (Smith and Read 1997; Marschner and Rengel 2007). Mycorrhizal functions include improvement of plant establishment, enhancement of nutrient uptake, protection against cultural and environmental stresses, and the improvement of soil structure (Barea 1997).

All types of ecological situations can be suitable for mycorrhizal symbiosis. Most plant species are able of forming this symbiosis naturally; the most common type involved in the normal cropping systems is the arbuscular mycorrhizal (AM) type (Smith and Read 1997). A very common group of soilborne fungal order, Glomales in the Zygomycetes, has this AM fungi (Morton and Redecker 2001).

Recent developments in molecular biology based on PCR-based approaches are being applied to the genetic characterization of AM fungi (Sanders et al. 1996). The analysis of rRNA sequences in last few years has demonstrated the polymorphism of these genes in AM fungi, particularly those corresponding to the small ribosomal subunit 18S, thus allowing for detailed diversity and phylogenetic studies (Redecker et al. 2000; Daniell et al. 2001). Novel techniques for microbial molecular ecology studies, like PCR-SSCP and PCR-TGGE, are being adopted for the characterization of different ecotypes of AM fungi, both in soil and in roots (Pawlowska and Taylor 2004).

3.1 Taxonomy of AM Fungi

Arbuscular mycorrhizal (AM) fungi form a widespread and ecologically important symbiosis with plants in the land ecosystem. The phylogeny of the largest presently accepted genus, *Glomus*, of the monogeneric family Glomaceae (Glomales; AM fungi) has been analyzed. Phylogenetic trees were computed from nearly full-length SSU

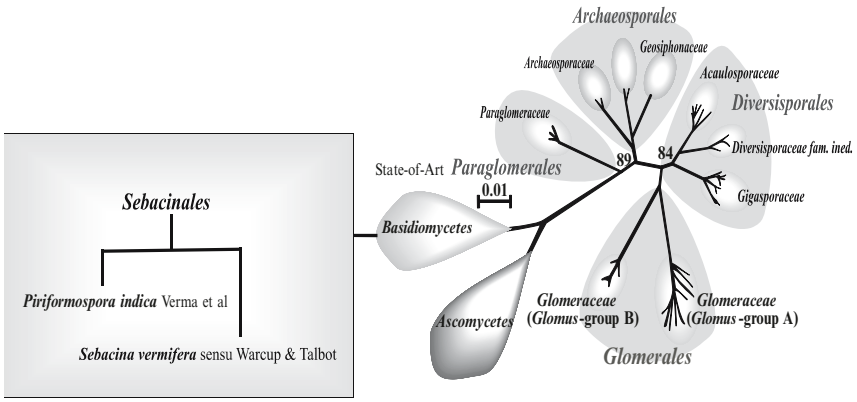

Fig. 1 Proposed generalised taxonomic structure of the AM and related fungi (Glomeromycota), based on SSU rRNA gene sequences. *Thick lines* delineate bootstrap support above 95%, lower values are given on the branches. The four-order structure for the phylogenetic position of *P. indica* (after Schüßler et al. 2001; Varma et al. 2001)

rRNA gene sequences of 30 isolates, and show that "*Glomus*" is not monophyletic. Even after the very recent separation of *Archaeospora* and *Paraglomus* from "*Glomus*" the genus further separates into two suprageneric clades. One of them diverges further into two subclades, differing by phylogenetic distances equivalent to family level. The other, comprising *Glomus versiforme*, *G. spurcum* and a species morphologically similar to *G. etunicatum*, is not closely related to the Glomaceae, but clusters together with the Acaulosporaceae and Gigasporaceae in a monophyletic clade. Based on the molecular evidence, a new family, separate from the Glomaceae, is required to accommodate this group of organisms, and initially named Diversisporaceae fam. Ined (Fig. 1). The current taxonomic concept of the recently erected family Archaeosporaceae also requires future emendation, because *Geosiphon pyriformis* (Geosiphonaceae) renders *Archaeospora***,** the sole genus formally included in this family, paraphyletic. The suborders Gigasporineae and Glominaeae are not congruent with the natural phylogeny of the AM fungi (Schüßler et al. 2001; Walker and Schüßler 2004).

3.2 Functions of AM Fungi

During the process of AM formation (Giovannetti 2000; Avio et al. 2006; Bedini et al. 2007), in which the plant "accepts" the fungal colonization without any significant rejection reaction (Dumas-Gaudot et al. 2000), a series of root–fungus interactions give way to the integration of both organisms. The establishment of the symbiosis is the result of a continuous molecular

"dialogue" between plant and fungus, as exerted through the exchange of both recognition and acceptance signals (Vierheilig and Piché 2002). The result of this dialogue finally depends on the genome expression of both partners (Gianinazzi-Pearson et al. 1996; Bestel-Corre et al. 2004; Franken and Requena 2001; Kuster et al. 2007).

After the biotrophic colonization of root cortex, these AM fungi develop an external mycelium which serves as a bridge connecting the root with the surrounding soil microhabitats. Such mycorrhizal (fungal–root) symbiosis is critical in nutrient cycling in soil–plant systems (Smith and Read 1997). In cooperation with other soil organisms, the external AM fungal mycelium forms water-stable aggregates necessary for good soil tilth. The AM symbiotic association helps in the improvement of plant health through increased protection against biotic and abiotic stresses (Miller and Jastrow 2000; Requena et al. 2001; Requena et al. 2007; Ocon et al. 2007). Some relevant papers dealing with interaction of mycorrhizal fungi with root pathogens are listed in Table 1.

3.3 Piriformospora indica: a Novel Mycorrhiza-like Fungus

The scientists from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have screened a novel endophytic root-colonizing fungus *Piriformospora indica* for the first time, which mimics the capabilities of a typical arbuscular mycorrhizal fungus. Based on the anatomical and genomic studies, *P. indica* has been attributed to highly evolved Hymenomycetes (Basidiomycetes). This fungus has been patented (Varma A & Franken P, 1997) at theEuropean Patent Office, Müenchen, Germany (Patent No. 97121440.8–2105, Nov. 1998/ WO 99/ 29177, June 17, 1999) and the culture has been deposited at Braunsweich, Germany (DMS No. 11827). The 18s rDNA fragment has been deposited in GenBank, Bethesda, USA. Like arbuscular mycorrhizal (AM) fungi, *P. indica* functions as bioregulator, biofertilizer and bioprotector, overcomes the water stress (dehydration), delays the wilting of the leaves, and prolongs aging of callus tissues. Interestingly, the host spectrum of *P. indica* is very much like AM fungi and can colonize the roots and improve the health, vigor and survival of a wide range of mono- and dicotyledon plants. This fungus mediates uptake of phosphorus from the substratum and its translocation to the host by an energy-dependent active process. It serves as a strong agent for biological hardening of tissue culture-raised plants, protecting them from "transplantation shock", rendering almost 100% survivals on the hosts tested. This fungus is also a potential biological agent against potent root pathogens. Thus, it displays immense potential to be utilized as biological tool for plant promotion, protection from pests, and for relieving stress conditions such as those caused due to acidity, desiccation and heavy metal toxicity (Sherameti et al. 2005; Shahollari et al. 2005; Waller et al. 2005; Serfling et al. 2007). Multifunctional aspect of the fungus is depicted in Fig. 2.

Table 1 Relevant publications

Title	Reference
Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soilborne fungal pathogens	Budi et al. (1999)
Arbuscular mycorrhizas: physiology and function	Linderman (2000)
What do root pathogens see in mycorrhizas?	James (2001
The defense response elicited by the pathogen *Rhizoctonia solani* is suppressed by colonization of the AM-fungus *Glomus intraradices*	Guenoune (2001)
Insect pathogens as biological control agents: do they have a future?	Lacey et al. (2001)
Microbial populations responsible for specific soil suppressiveness to plant pathogens	Weller et al. (2002)
Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato	Diedhiou et al. (2003)
A review of fungal antagonists of powdery mildews and their potential as biocontrol agents	Kiss (2003)
Assessment of arbuscular mycorrhizal fungi as potential biocontrol agents for *Poa annua* L. in fine turf	Gange et al. (2004)
Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness	Garbeva et al. (2004)
The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems	Harrier and Watson (2004)
Arbuscular mycorrhizal fungi reduce development of pea root-rot caused by *Aphanomyces euteiches* using oospores as pathogen inoculum	Thygesen et al. (2004)
Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects	Compant et al. (2005)
Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against *Verticillium*-induced wilt in pepper	Garmendia et al. (2005)
The endophytic fungus *Piriformospora indica* reprograms barley to salt stress tolerance, disease resistance and higher yield	Waller et al. (2005)
Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203	Shoresh et al. (2005)
Biological control of root-rot disease complex of chickpea by AM fungi	Siddiqui et al. (2006)
Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (Vitis amurensis Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3	Li et al. (2006)
Biological control of plant pathogens	Pal et al. (2006)
Root exudates of mycorrhizal tomato plants exhibit a different effect on microconidia germination of *Fusarium oxysporum* f. sp. lycopersici than root exudates from non-mycorrhizal tomato plants	Scheffknecht et al. (2006)
Biological control of plant diseases	Chincholkar and Mukerji (2007)
Mycorrhizae in the integrated pest and disease management	Mukerji and Ciancio (2007)
Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions	Serfling et al. (2007)
Plant biology: jasmonate perception machines	Farmer (2007)

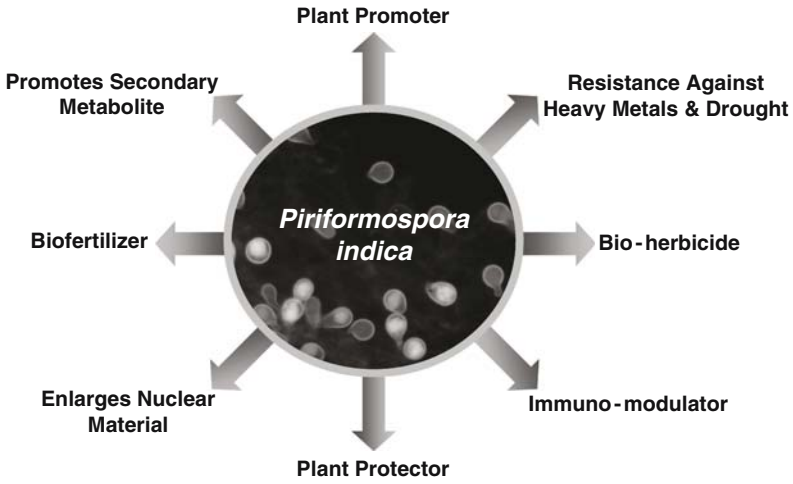

Fig. 2 The multifunctional aspect of *Piriformospora indica*

4 Plant Growth-promoting Rhizobacteria (PGPRs)

The beneficial root-colonizing rhizosphere bacteria, the plant growth-promoting rhizobacteria (PGPRs), are defined by three intrinsic characteristics (1) they have to survive and multiply in microhabitats associated to root surface, in competition with native microbiota, at least for the time needed to express their plant promotion activities, (2) they must have the ability for root colonization, and (3) they must have the ability for plant growth promotion (Kloepper 1994). The PGPRs are known to carry out many important ecosystem processes, such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment (Lemanceau and Alabouvette 1993; Glick 1995; Broek and Vanderleyden 1995; Probanza et al. 2002; Baldock et al. 2004). Many bacterial taxa include PGPR strains. *Pseudomonas* and *Bacillus* are the most common described genera as possessing species with PGPRs ability, and some strains from these and other genera are being used as seed inoculants (Bertrand et al. 2001; Ziedan 2006).

Azospirillum sp. is considered PGPR (Bashan 1999; Bashan and Gonzalez 1999; El Zemrany et al. 2006) and used as seed inoculants under field conditions (Dobbelaere et al. 2001). The main activity of these bacteria is associated to the production of auxin-type phytohormones (Dobbelaere et al. 1999). The production and significance of auxins have been investigated at a molecular level (van de Broek et al. 1999; Lambrecht et al. 2000). The molecular bases of the biocontrol ability of these rhizobacteria are currently being investigated, and systemic-induced resistance has been argued as a mechanism of disease suppression by endophytes (Duijff et al. 1998; Ramarathnam and Dilantha 2006) or other PGPRs (Defago and Keel 1995; Chin-A-Woeng et al. 2001; Compant et al. 2005).

5 Biological Control

The term "biological control" has been used in different fields of biology. In plant pathology, the term applies to the use of microbial antagonists to suppress diseases as well as the use of host-specific pathogens to control weed populations. In both fields, the organism that suppresses the pest or pathogen is referred to as the biological control agent (BCA).

5.1 Basics of Biocontrol

Biological control is the suppression of damaging activities of one organism by one or more other organisms. This refers to the purposeful utilization of introduced or resident living organisms, other than disease-resistant host plants, to suppress the activities and populations of one or more plant pathogens. This may involve the use of microbial inoculants to suppress a single type or class of plant diseases. Or, this may involve managing soils to promote the combined activities of native soil- and plant-associated organisms that contribute to general suppression. Finally, biological control refers to the suppression of a single pathogen (or pest), by a single antagonist in a single cropping system. With regards to plant diseases, suppression can be accomplished in many ways (Pal and Gardener 2006).

5.2 Biocontrol Versus Chemical Control

The use of chemical pesticides and insecticides based on synthetic formulations with a higher specificity towards their target organism has led to dramatic improvements in the production of crop plants, provided a reliable supply of cheap food, generally exhibited low overall toxicity and with little immediate impact on the environment. But the long-term effects of these compounds have a detrimental effect on soil fertility and development of resistance in pathogens. Excessive use of these chemicals has an effect on food quality and environmental damage leading to nonsustainability of farming. The need of the day is to develop sustainable, long-term, and ecological- and environment-friendly alternatives to pesticides, which are naturally occurring microbes provided by nature for ages but not utilized to their full potential.

The most obvious and apparently biological control is a potent means of reducing the damage caused by plant pathogens. The potential agents for biocontrol activity are rhizosphere-competent fungi and bacteria which, in addition to their antagonistic activity, are capable of inducing growth responses by either controlling minor pathogens or by producing growth-stimulating factors.

6 Microbial Diversity and Disease Suppression

Plants are surrounded by diverse types of microbial organisms, which contribute to biological control of plant diseases. Microbes contributing to the disease control can be classified as competitive saprophytes, facultative plant symbionts and facultative hyperparasites. These generally survive on dead plant material and are able to colonize and express biocontrol activities while growing on plant tissues. A few, like avirulent *Fusarium oxysporum* and binucleate *Rhizoctonia*-like fungi, are phylogenetically very similar to plant pathogens, but lack active virulence determinants for many of the plant hosts from which they can be recovered. Others, like *Pythium oligandrum* are currently classified as distinct species. *P. indica* blocks the mycelial development in *Gaeumannomyces graminis* and *Fusarium* sp. (Figs. 3 and 4). However, most are phylogenetically distinct from pathogens and, most often; they are subspecies variants of the same microbial groups. At the moment we do not have knowledge for the mechanism and the Biomolecules involved for imparting such prominent inhibitory impact.

Due to the ease with which they can be cultured, most biocontrol research has focused on a limited number of bacterial (*Bacillus, Burkholderia, Lysobacter,*

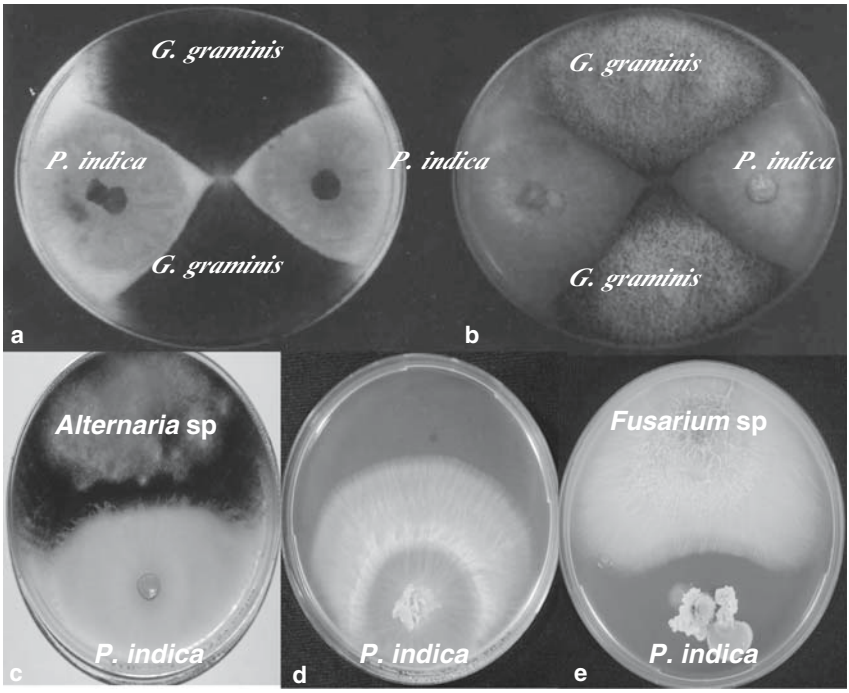

Fig. 3 Interaction of *P. indica* with plant pathogens. **a** *Gaeumannomyces graminis* (front view). **b** *G. graminis* (back view). **c** *Alternaria* sp. **d** *P. indica* (control). **e** *Fusarium* sp. (partially modified from Varma et al. 2001)

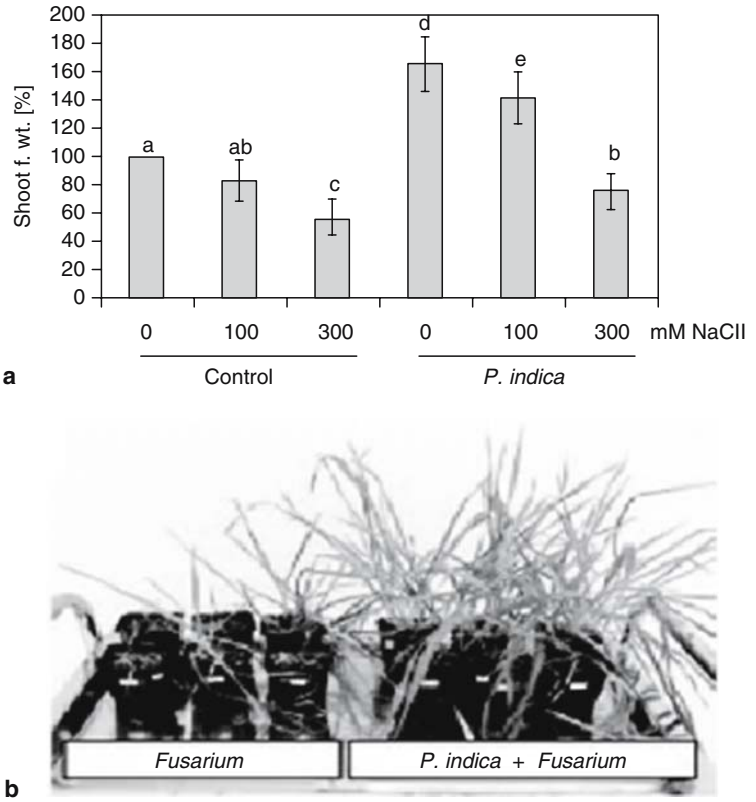

Fig. 4 Impact of *P. indica* on salt-stress tolerance and root infections by *F. culmorum*. **a** Shoot fresh weight of *P. indica* and control (noninfested) plants was determined in 5-week-old plants that had been grown for the final 2 weeks in the presence of 100 or 300 mM NaCl, in hydroponic culture. Data points are representative of three independent experiments. *Error bars* SD. **b** Plant phenotypes demonstrating the protective potential of *P. indica* toward *F. culmorum* (Waller et al. 2005)

Pantoea, Pseudomonas, and *Streptomyces*) and fungal (*Ampelomyces, Coniothyrium, Dactylella, Gliocladium, Paecilomyces* and *Trichoderma*) genera. Still, other microbes that are more recalcitrant to in vitro culturing have been intensively studied. These include mycorrhizal fungi, e.g., *Pisolithus* and *Glomus* spp. that can limit subsequent infections, and some hyperparasites of plant pathogens, e.g., *Pasteuria penetrans,* which attacks root-knot nematodes. Because multiple infections can and do take place in field-grown plants, weakly virulent pathogens can contribute to the suppression of more virulent pathogens, via the induction of host defenses. Lastly, there are the many general micro- and mesofauna predators, such as protists, *Collembola*, mites, nematodes, annelids, and insect larvae, whose activities can reduce pathogen biomass but may also facilitate infection and/or stimulate plant host defenses by virtue of their own herbivorous activities.

While various epiphytes and endophytes may contribute to biological control, the ubiquity of mycorrhizae deserves special consideration. Mycorrhizae are formed as the result of mutualist symbioses between fungi and plants and occur on most plant species. Because they are formed early in the development of the plants, they represent nearly ubiquitous root colonists that assist plants with the uptake of nutrients (especially phosphorus and micronutrients). The arbuscular mycorrhizal fungi (AM, also known as arbuscular mycorrhizal or endomycorrhizal fungi) are all members of the zygomycota, and the current classification contains one order, the Glomales, encompassing six genera into which 149 species have been classified (Morton and Benny 1990; Garbewa et al. 2004; Mukerji and Ciancio 2007). Arbuscular mycorrhizae involve aseptate fungi and are named for characteristic structures like arbuscles and vesicles found in the root cortex. Arbuscules start to form by repeated dichotomous branching of fungal hyphae approximately 2 days after root penetration inside the root cortical cell. Arbuscules are believed to be the site of communication between the host and the fungus. Vesicles are basically hyphal swellings in the root cortex that contain lipids and cytoplasm and act as storage organ of AM. These structures may present intra- and intercellular and can often develop thick walls in older roots. These thick-walled structures may function as propagules (Biermann and Linderman 1983). During colonization, AM fungi can prevent root infections by reducing the access sites and stimulating host defense. AM fungi have been found to reduce the incidence of root-knot nematodes (Linderman 1994). Various mechanisms also allow AM fungi to increase a plant's stress tolerance. This includes the intricate network of fungal hyphae around the roots which block pathogen infections. Inoculation of apple tree seedlings with the AM fungi *Glomus fasciculatum* and *G. macrocarpum* suppressed apple replant disease caused by phytotoxic myxomycetes (Catska 1994). AM fungi protect the host plant against root-infecting pathogenic bacteria. The damage due to *Pseudomonas syringae* on tomato may be significantly reduced when the plants are well-colonized by mycorrhizae (Garcia-Garrido and Ocampo 1989; Garcia-Garrido et al. 2000). The mechanisms involved in these interactions include physical protection, chemical interactions and indirect effects (Fitter and Garbaye 1994). The other mechanisms employed by AM fungi to indirectly suppress plant pathogens include enhanced nutrition to plants, morphological changes in the root by increased lignification, changes in the chemical composition of the plant tissues like antifungal chitinase, isoflavonoids, etc. (Morris and Ward 1992; Garcia-Garrido et al. 2000); alleviation of abiotic stress, salinity stress (Rabie and Almadini 2005), and changes in the microbial composition in the mycorrhizosphere (Linderman 1994). In contrast to AM fungi, ectomycorrhizae proliferate outside the root surface and form a sheath around the root by the combination of mass of root and hyphae called a mantle. Disease protection by ectomycorrhizal fungi may involve multiple mechanisms including antibiosis, synthesis of fungistatic compounds by plant roots in response to mycorrhizal infection and a physical barrier of the fungal mantle around the plant root (Duchesne 1994; Garcia-Garrido et al. 2000). Ectomycorrhizal fungi like *Paxillus involutus* effectively controlled root rot caused by *Fusarium oxysporum* and *Fusarium moniliforme* in red pine. Inoculation of sand pine with *Pisolithus*

tinctorius, another ectomycorrhizal fungus, controlled disease caused by *Phytophthora cinnamomi* (Ross and Marx 1972).

Since plant diseases may be suppressed by the activities of one or more plant-associated microbes, attempts have been made to characterize the organisms involved in biological control. Historically, this has been done primarily through isolation, characterization, and application of individual organisms. By design, this approach focuses on specific forms of disease suppression. Specific suppression results from the activities of one or just a few microbial antagonists. This type of suppression is thought to be occurring when inoculation of a biocontrol agent results in substantial levels of disease suppressiveness. It may also occur in natural systems from time to time. For example, the introduction of *Pseudomonas fluorescens* that produce the antibiotic 2,4-diacetylphloroglucinol can result in the suppression of various soil borne pathogens. However, specific agents must compete with other soil- and root-associated microbes to survive, propagate, and express their antagonistic potential during those times when the targeted pathogens pose an active threat to plant health. In contrast, general suppression is more frequently invoked to explain the reduced incidence or severity of plant diseases because the activities of multiple organisms can contribute to a reduction in disease pressure. High soil organic matter supports a large and diverse mass of microbes resulting in the availability of fewer ecological niches for which a pathogen competes. The extent of general suppression will vary substantially depending on the quantity and quality of organic matter present in a soil (Hoitink and Boehm 1999). Functional redundancy within different microbial communities allows for rapid depletion of the available soil nutrient pool under a large variety of conditions, before the pathogens can utilize them to proliferate and cause disease. For example, diverse seed-colonizing bacteria can consume nutrients that are released into the soil during germination, thereby suppressing pathogen germination and growth (McKellar and Nelson 2003). Manipulation of agricultural systems, through additions of composts, green manures and cover crops, is aimed at in proving endogenous levels of general suppression (Hameeda et al. 2007).

7 Interactions for the Biological Control of Root Pathogens

Once the AM status has been established in plant roots, a reduced damage caused by soilborne plant pathogens has been shown by several mechanisms suggested to explain the enhancement of plant resistance/tolerance in mycorrhizal plants (Azcón-Aguilar and Barea 1992, 1996; Linderman 1994, 2000). The important one is based on the microbial changes produced in the mycorrhizosphere (Mukerji et al. 1998; Giri et al. 2004). Some microbial shifts occur which result in microbial equilibria and influence the growth and health of the plants. Although this effect has not been assessed specifically as a mechanism for AM-associated biological control, there are indications that such a mechanism can be involved (Azcón-Aguilar and Barea 1992, 1996; Linderman 1994, 2000). In any case, it has been demonstrated that such an effect is dependent on the AM fungus

involved, as well as the substrate and host plant (Azcón-Aguilar and Barea 1996; Linderman 2000).

Since specific PGPR antagonistic to root pathogens are being used as biological control agents (Alabouvette et al. 1997), it has been proposed to exploit the prophylactic ability of AM fungi in association with these antagonists (Azcón-Aguilar and Barea 1996; Linderman 1994, 2000).

Several studies have demonstrated that microbial antagonists of fungal pathogens, either fungi or PGPRs, do not exert any antimicrobial effect against AM fungi. This is a key point to exploit the possibilities of dual (AM fungi and PGPRs) inoculation in plant defense against root pathogens.

7.1 Types of Interactions Contributing to Biological Control

The interactions with various organisms throughout their lifecycle affect plant health in various ways. The mechanisms of biological control depend upon these various ways that organisms interact. The mode of interaction is by means of direct or indirect contact. The types of interactions were referred to as mutualism, protocooperation, commensalism, neutralism, competition, parasitism, and predation (Odum 1953). Since, both the plants and microbes are involved in the development of plant diseases, the interactions leading to biological control occur at multiple levels of scale.

Biological control is considered a net positive result on the plants arising from a variety of specific and non-specific interactions (Pal and Gardener 2006). An association between two or more species where both species derive benefit is known as *mutualism*. It is an obligatory interaction which involves close physical and biochemical contact, like those between plants and mycorrhizal fungi. However, they are generally facultative and opportunistic. *Rhizobium* can reproduce either in the soil or through their mutualistic association with legume plants. This interaction can contribute to biological control, by fortifying the plant with improved nutrition and/or by stimulating host defenses. Protocooperation is a form of mutualism, but the involved organisms do not depend exclusively on each other for survival. Since these are independent of any specific host, disease suppression varies depending on environmental conditions. Further, commensalism is a symbiotic interaction, where one organism benefits and the other is neither harmed nor benefited. The plant-associated microbes are mostly assumed to be commensals with regards to the host plant. Neutralism describes the interactions when the population density of one species has absolutely no effect on the other. With reference to biological control, an inability to associate the population dynamics of pathogen with that of another organism would indicate neutralism. In contrast, *antagonism* between organisms results in a negative outcome for one or both. Competition within and between species results in decreased growth, activity and/or fecundity of the interacting organisms. Biocontrol can occur when nonpathogens compete with pathogens for nutrients in and around the host plant.

Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Parasitism is a symbiosis in which two phylogenetically unrelated organisms coexist over a prolonged period of time. In this type of association, one organism, the parasite benefits and the other, the host is harmed. The activities of various hyperparasites, i.e., agents that parasitize plant pathogens, can result in biocontrol. Lastly, *predation* refers to killing of one organism by another for consumption and sustenance. The term has been applied to the actions of microbes that consume pathogen biomass for sustenance. Biological control results from all of these types of interactions, depending on the environmental context within which they occur. Significant biological control, as defined above, most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens (Pal and Gardener 2006).

7.2 Mechanisms of Biological Control

Biological control can result from many different types of interactions between organisms; the mechanisms operating in different experimental situations are to be characterized. The pathogens are antagonized by the presence and activities of other organisms in vicinity. Direct antagonism results from physical contact and/or a high-degree of selectivity for the pathogen by the mechanisms expressed by the BCAs. In such a scheme, hyperparasitism by obligate parasites of a plant pathogen would be considered the most direct type of antagonism because the activities of no other organism would be required to exert a suppressive effect. In contrast, indirect antagonisms result from activities that do not involve sensing or targeting a pathogen by the BCA(s). Antagonistic pathways are as depicted in Table 2. Stimulation of plant host defense pathways by nonpathogenic BCAs is the most indirect form of antagonism. The most effective BCAs studied to date appear to antagonize pathogens using multiple mechanisms. For instance, pseudomonad's known to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) may also induce host defenses (Iavicoli et al. 2003). Additionally, DAPG-producers can aggressively colonize roots, a trait that might further contribute to their ability to suppress pathogen activity in the rhizosphere of wheat through competition for organic nutrients (Weller et al. 2002).

7.2.1 Hyperparasites and Predation

In general, there are four major classes of hyperparasites: obligate bacterial pathogens, hypoviruses, facultative parasites, and predators. In hyperparasitism, the pathogen is directly attacked by a specific BCA that kills it or its propagules.

Table 2 Antagonism responsible for biological control of plant pathogens

Type	Mechanism adopted
Direct antagonism	Hyperparasitism/predation
	Examples: lytic/some nonlytic mycoviruses *Lysobacter enzymogenes, Pasteuria penetrans, Trichoderma virens*
Mixed-path antagonism	Antibiotics
	Mode: 2,4 diacetylphloroglucinol, phenazines, cyclic lipopeptides
	Lytic enzymes
	Examples: chitinases, glucanases, proteases
	Unregulated waste products
	Examples: ammonia, carbon dioxide, hydrogen cyanide
Physical/chemical interference	
Indirect antagonism	Competition
	Mode: exudates/leachates consumption, siderophore scavenging, physical niche occupation
	Induction of host resistance
	Mode: contact with fungal cell walls, detection of pathogen-associated molecular patterns, phytohormone-mediated induction

Modified from Pal and Gardener (2006)

Pasteuria penetrans is an obligate bacterial pathogen of root-knot nematodes that has been used as a BCA. There are several fungal parasites of plant pathogens, including those that attack sclerotia (e.g., *Coniothyrium minitans*) while others attack living hyphae (e.g., *Pythium oligandrum*). And, a single fungal pathogen can be attacked by multiple hyperparasites. For example, *Acremonium alternatum, Acrodontium crateriforme, Ampelomyces quisqualis, Cladosporium oxysporum*, and *Gliocladium virens* are just a few of the fungi that have the capacity to parasitize powdery mildew pathogens (Kiss 2003). Other hyperparasites attack plant-pathogenic nematodes during different stages of their life cycles (e.g., *Paecilomyces lilacinus* and *Dactylella oviparasitica*). In contrast to hyperparasitism, microbial predation is more general and pathogen nonspecific and generally provides less predictable levels of disease control. Some BCAs exhibit predatory behavior under nutrient-limited conditions. However, *Trichoderma* produce a range of enzymes that are directed against cell walls of fungi (Benhamou and Chet 1997).

7.2.2 Antibiotic-mediated Suppression

Microbial toxins that kill other microorganisms at low concentrations are known as antibiotics. Most microbes produce and secrete one or more compounds with

Table 3 Antibiotics produced by Biological Control Agents (BCA)

Organisms	Antibiotics produced	Target pathogen
Pseudomonas fluorescens	2,4-diacetyl-phloroglucinol	*Pythium* sp.
	Phenazines	*Gaeumannomyces graminis* var. *tritici*
	Pyoluteorin, Pyrrolnitrin	*Pythium ultimum Rhizoctonia solani*
Agrobacterium radiobacter	Agrocin 84	*Agrobacterium tumefaciens*
Bacillus subtilis	Bacillomycin D	*Aspergillus flavus*
	Iturin A	*Botrytis cinerea R. solani*
	Mycosubtilin	*Pythium aphanidermatum*
Bacillus cereus	Zwittermicin A	*Phytophthora medicaginis* *P. aphanidermatum*
Bacillus amyloliquefaciens	Bacillomycin, Fengycin	*Fusarium oxysporum*
Lysobacter sp.	Xanthobaccin A	*Aphanomyces cochlioides*
Trichoderma virens	Gliotoxin	*R. solani*

Modified from Pal and Gardener (2006)

antibiotic activity. In some instances, antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens and the diseases they cause. Some examples of antibiotics reported to be involved in plant pathogen suppression are listed in Table 3. In all cases, the antibiotics have been shown to be particularly effective at suppressing growth of the target pathogen in vitro and/or in situ. Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. The ability to produce multiple antibiotics probably helps to suppress diverse microbial competitors, some of which are likely to be plant pathogens. The ability to produce multiple classes of antibiotics, that differentially inhibit different pathogens, is likely to enhance biological control. More recently, *Pseudomonas putida* WCS358r strains genetically engineered to produce phenazine and DAPG (2,4-diacetyl-phloroglucinol) displayed improved capacities to suppress plant diseases in field-grown wheat (Glandorf et al. 2001).

7.2.3 Lytic Enzymes and Other Byproducts of Microbial Life

Many microorganisms produce and release lytic enzymes that can hydrolyze a wide variety of polymeric compounds, including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly. For example, control of *Sclerotium rolfsii* by *Serratia marcescens* appeared to be mediated by chitinase expression (Ordentlich et al. 1988). And, a b-1,3-glucanase contributes significantly to biocontrol activities of *Lysobacter enzymogenes* strain C_3 (Palumbo et al. 2005). While they may stress and/or lyse cell walls of living organisms, these enzymes generally act to decompose plant residues and nonliving organic matter. Microbes showing a preference for colonizing and lysing plant

pathogens are classified as biocontrol agents. *Lysobacter* and Myxobacteria are known to produce lytic enzymes, and some isolates have been shown to be effective at suppressing fungal plant pathogens.

7.2.4 Competition

From a microbial perspective, soils and living plant surfaces are frequently nutrient-limited environments. To successfully colonize the phytosphere, a microbe must effectively compete for the available nutrients. On plant surfaces, host-supplied nutrients include exudates, leachates, or senesced tissue. In general, soilborne pathogens, such as species of *Fusarium* and *Pythium* that infect through mycelial contact, are more susceptible to competition from other soil- and plant-associated microbes than those pathogens that germinate directly on plant surfaces and infect through appressoria and infection pegs. The most abundant nonpathogenic plant-associated microbes are generally thought to protect the plant by rapid colonization and thereby exhausting the limited available substrates so that none are available for pathogens to grow. At the same time, these microbes produce metabolites that suppress pathogens. These microbes colonize the sites where water and carbon-containing nutrients are most readily available, such as exit points of secondary roots, damaged epidermal cells, and nectarines, and utilize the root mucilage.

7.2.5 Induction of Host Resistance

There are numerous environmental stimuli, including gravity, light, temperature, physical stress, water and nutrient availability, to which plants actively respond. Plants also respond to a variety of chemical stimuli produced by soil- and plant-associated microbes which either induce or condition plant host defenses through biochemical changes that enhance resistance against subsequent infection by a variety of pathogens. Recently, characterization of the determinants and pathways of induced resistance stimulated by biological control agents and other nonpathogenic microbes have been studied as stated in Table 4. The first of these pathways, termed systemic acquired resistance (SAR), is mediated by salicylic acid (SA), a compound which is frequently produced following pathogen infection, and typically leads to the expression of pathogenesis-related (PR) proteins. These PR proteins include a variety of enzymes some of which may act directly to lyse the invading cells, reinforce cell wall boundaries to resist infections, or induce localized cell death. A second phenotype, first referred to as induced systemic resistance (ISR), is mediated by jasmonic acid (JA) and/or ethylene, which are produced following applications of some nonpathogenic rhizobacteria. Interestingly, the SA- and JA- dependent defense pathways can be mutually antagonistic, and some bacterial pathogens take advantage of this to overcome the SAR. For example, pathogenic strains of *Pseudomonas syringae* produce coronatine, which is similar to JA, to overcome the SA-mediated pathway (He et al. 2004). Because the various host-resistance pathways can be activated to

Table 4 Bacterial determinants and types of host resistance induced by biocontrol agents

Strains	Hosts	Bacterial determinants	Type
Bacillus mycoides	*Beta vulgaris*	Peroxidase, chitinase and β-1,3-glucanase	ISR
Bacillus subtilis	*Arabidopsis*	2,3-butanediol	ISR
Pseudomonas fluorescens	*Nicotiana tabacum*	Siderophore	SAR
	Arabidopsis	Antibiotics (DAPG)	ISR
	Raphanus sativus	Lipopolysaccharide	ISR
		Siderophore	
		Iron-regulated factor	
	Dianthus caryophyllus	Lipopolysaccharide	ISR
	Raphanus sativus	Lipopolysaccharide	ISR
		Iron regulated factor	
	Arabidopsis	Lipopolysaccharide	ISR
	Solanum lycopersicum	Lipopolysaccharide	ISR
Pseudomonas putida	*Arabidopsis*	Lipopolysaccharide	ISR
		Siderophore	ISR
	Bean	Z,3-hexenal	ISR
Serratia marcescens	*Cucumis sativus*	Siderophore	ISR

Modified from Pal and Gardener (2006)

varying degrees by different microbes and insect feeding, it is plausible that multiple stimuli are constantly being received and processed by the plant. Thus, the magnitude and duration of host defense induction will likely vary over time. Only if induction can be controlled, i.e., by overwhelming or synergistically interacting with endogenous signals, will host resistance be increased.

A number of strains of root-colonizing microbes have been identified as potential elicitors of plant host defenses. Some biocontrol strains of *Pseudomonas* sp. and *Trichoderma* sp. are known to strongly induce plant host defenses (Haas et al. 1991; Harman et al. 2004). In several instances, inoculations with plant-growth-promoting rhizobacteria (PGPR) were effective in controlling multiple diseases caused by different pathogens, including anthracnose (*Colletotrichum lagenarium*), angular leaf spot (*Pseudomonas syringae* pv. *lachrymans* and bacterial wilt (*Erwinia tracheiphila*). A number of chemical elicitors of SAR and ISR may be produced by the PGPR strains upon inoculation, including salicylic acid, siderophore, lipopolysaccharides, and 2,3-butanediol, and other volatile substances (Van Loon et al. 1998; Ongena et al. 2004; Varma and Chincholkar 2007). Again, there may be multiple functions to such molecules blurring the lines between direct and indirect antagonisms. More generally, a substantial number of microbial products have been identified as elicitors of host defenses, indicating that host defenses are likely stimulated continually over the course of a plant's lifecycle. Excluding the components directly related to pathogenesis, these inducers include: lipopolysaccharides and flagellin from Gram-negative bacteri; cold shock proteins of diverse bacteria; transglutaminase, elicitins, and β-glucans in Oomycetes; invertase in yeast; chitin and ergosterol in all fungi; and xylanase in *Trichoderma* (Numberger et al. 2004). These

data suggest that plants would detect the composition of their plant-associated microbial communities and respond to changes in the abundance, types, and localization of many different signals. The importance of such interactions is indicated by the fact that further induction of host resistance pathways, by chemical and microbiological inducers, is not always effective at improving plant health or productivity in the field.

8 Biocontrol Research, Development and Adoption

Much has been learned from the biological control research conducted over the past 40 years. But, in addition to learning the lessons of the past, biocontrol researchers need to look forward to define new and different questions, the answers to which will help facilitate new biocontrol technologies and applications. Currently, fundamental advances in computing, molecular biology, analytical chemistry, and statistics have led to new research aimed at characterizing the structure and functions of biocontrol agents, pathogens, and host plants at the molecular, cellular, organismal, and ecological levels.

Growers are interested in reducing dependence on chemical inputs, so biological controls (defined in the narrow sense) can be expected to play an important role in Integrated Pest Management (IPM) systems. To deploy biorational controls of insect pests and diseases these include BCAs, introduced as inoculants or amendments, as well as active ingredients directly derived from natural origins and having a low impact on the environment and nontarget organisms, is the basic theme behind it.

9 Conclusions

In general, though, regulatory and cultural concerns about the health and safety of specific classes of pesticides are the primary economic drivers promoting the adoption of biological control strategies in urban and rural landscapes. Self-perpetuating biological controls (e.g., hypovirulence of the chestnut blight pathogen) are also needed for control of diseases in forested and rangeland ecosystems where high application rates over larger land areas are not economically-feasible. In terms of efficacy and reliability, the greatest successes in biological control have been achieved in situations where environmental conditions are most controlled or predictable and where biocontrol agents can preemptively colonize the infection court. Monocyclic, soil borne and post-harvest diseases have been controlled effectively by biological control agents that act as bioprotectants (i.e., preventing infections). Specific applications for high value crops targeting specific diseases (e.g., fireblight, downy mildew, and several nematode diseases) have also been adopted. There is considerable interest in the exploitation of microbial biological control agents (MBCAs) for the control of crop pests, weeds and diseases. MBCAs can be

used where chemical pesticides are banned or being phased out or where pests have developed resistance to standard chemicals. The use of MBCAs can play an important role in crop protection, as a key element in integrated pest management (IPM) programmes. However, despite considerable research efforts on the development of new biological control agents, the number of such products on the market is still extremely low. In areas that previously constrained the commercialization of MBCAs, discovery, fermentation, formulation and application, significant progress has been made. Initiatives by stakeholders from industry, science, regulatory authorities, policy and environment are underway to accelerate market introduction of MBCAs. As research unravels the various conditions needed for successful biocontrol of different diseases, the adoption of MBCAs in IPM systems is bound to increase in the years ahead.

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References

Alabouvette C, Schippers B, Lemanceau P, Bakker PAHM (1997) Biological control of *Fusarium*-wilts: towards development of commercial product. In: Boland G J, Kuykendall L D (eds) Plant microbe interactions and biological control. Dekker, New York, pp 15–36

Avio L, Pellegrino E, Bonari E, Giovannetti M (2006) Functional diversity of arbuscular mycorrhizal fungal isolates in relation to extraradical mycelial networks. New Phytol 172:347–357

Azcón-Aguilar C, Barea JM (1992) Interactions between mycorrhizal fungi and other rhizosphere microorganisms. In: Allen MJ (eds) Mycorrhizal functioning. An integrative plant-fungal process. Routledge, Chapman & Hall, New York, pp 163–198

Azcón-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens. An overview of the mechanisms involved. Mycorrhiza 6:457–464

Bacon CW (1993) Abiotic stress tolerances (moisture and nutrients) and photosynthesis in endophyte-infected tall fescue. Agric Ecosyst Environ 44:123–141

Baldock JA, Masiello CA, Gélinas Y, Hedges J I (2004) Cycling and composition of organic matter in terrestrial and marine ecosystems. Mar Chem 92:39–64

Barea J M (1997) Mycorrhiza/bacteria interactions on plant growth promotion. In:Ogoshi A, Kobayashi L, Homma Y, Kodama F, Kondon N, Akino S (eds) Plant growth-promoting rhizobacteria, present status and future prospects. OECD, Paris, pp 150–158

Bashan Y (1999) Interactions of *Azospirillum* spp. in soils: a review. Biol Fertil Soils 29:246–256

Bashan Y, Gonzalez LE (1999) Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. Appl Microbiol Biotechnol 51:262–266

Bedini S, Avio L, Argese E, Giovannetti M (2007) Effects of long-term land use on arbuscular mycorrhizal fungi and glomalin-related soil protein. Agric Ecosys Environ 120:463–466

Benhamou N, Chet I (1997) Cellular and molecular mechanisms involved in the intersection between *Trichoderma harzianum* and *Pythium ultimum*. Appl Environ Microbiol 63:2095–2099

Bertrand H, Nalin R, Bally R, Cleyet-Marel JC (2001) Isolation and identification of the most efficient plant growth-promoting bacteria associated with canola (*Brassica napus*). Biol Fert Soils 33:152–156

Bestel-Corre G, Dumas-Gaudot E, and Gianinazzi S (2004) Proteomics as a tool to monitor plant-microbe endosymbioses in the rhizosphere. Mycorrhiza 14:1–10

Biermann B, Linderman RG (1983) Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol 95:97–105

Bills GF, Polishook JD (1992) Recovery of endophytic fungi from *Chamaecyparis thyroides*. Sydowia 44:1–12

Biological Control of Plant Diseases (2007) Eds. S. Chincholkar and K.G Mukerji. The Haworth Press

Boddy L, Griffith GS (1989) Role of endophytes and latent invasion in the development of decay communities in sapwood of angiospermous trees. Sydowia 41:41–73

Broek AV, Vanderleyden J (1995) Genetics of the *Azospirillum*-plant root association. Crit Rev Plant Sci 14:445–466

Brunner F, Petrini O (1992) Taxonomic studies of *Xylaria* species and xylariaceous endophytes by isozyme electrophoresis. Mycol Res 96:723–733

Budi SW, van Tuinen D, Martinotti G, Gianinazzi S (1999) Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soilborne fungal pathogens. Appl Environ Microbiol 65:5148–5150

Bultman TL, Murphy JC (2000) Do fungal endophytes mediate wound-induced resistance? In: Bacon CW, White JF (eds) Microbial endophytes. Dekker, New York, pp 421–452

Carroll G (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69:2–9

Catska V (1994) Interrelationship between vesicular-arbuscular mycorrhiza and rhizosphere microflora in apple replant disease. Biol Plant 36:99–104

Chin-A-Woeng TFC, Thomas-Oates JE, Lugtenberg BJJ, Bloemberg GV (2001). Introduction of the phzH gene of *Pseudomonas chlororaphis* PCL1391extends the range of biocontrol ability of phenazine1carboxylic acid producing *Pseudomonas*. Mol Plant Microb Interact 14:1006–1015

Clay K (1987) Effect of fungal endophytes on the seed and seedling biology of *Lolium perenne* and *Festuca arundinaceae*. Oecologia 73:358–362

Clay K (1990) Fungal endophytes of grasses. Annu Rev Ecol Syst 21:275–297

Clay K (1991) Fungal endophytes, grasses and herbivores. In: Barbosa P, Krischik VA, Jones CG (eds) Microbial mediation of plant herbivore interactions. Wiley, New York, pp 199–226

Compant S, Duffy B, Nowak J, Clément C, Barka E A (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959

Dahlman DL, Eichenseer H, Siegel MR (1991) Chemical perspective on endophyte grass interactions and their implications to insect herbivory. In: Barbosa P, Krischik VA, Jones CG (eds) Microbial mediation of plant herbivore interactions. Wiley, New York, pp 227–252

Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol Ecol 36:203–209

De Bary A (1866) Morphologie und Physiologi der Pilze, Flechten, und Myxomyceten. [Hofmeister's Handbook of Physiological Botany. Vol. 2.] Leipzig

Defago G, Keel C (1995) Pseudomonads as biocontrol agents of diseases caused by soilborne pathogens In: Hokkanen HMT, Lynch JM (eds) Benefits and risks of introducing biocontrol agents. Cambridge University Press, Cambridge

Diedhiou PM, Hallmann J, Oerke EC, Dehne HW (2003) Effects of arbuscular mycorrhizal fungi and a non-pathogenic *Fusarium oxysporum* on *Meloidogyne incognita* infestation of tomato. Mycorrhiza 13:199–204

Dobbelaere S, Croonenborghs A, Thys A, Vande Browk A, Vanderleyden J (1999) Phytostimulatory effect *Azospirillum brasilense* strains and auxins on wheat. Plant Soil 212:155–164

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto P, Labandera-Gonzalez C, Caballero-Mellado J, Aguirre JF, Kapulnik Y, Brener S, Burdman S, Kadouri D, Sarig S, Okon Y (2001) Response of agronomically important crops to inoculation with *Azospirillum*. Aust J Plant Physiol 28:1–9

Duchesne LC (1994) Role of ectomycorrhizal fungi in biocontrol. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, St. Paul, Minn., pp 27–45

Duijff BJ, Pouhair D, Olivain C, Alabouvette C, Lemanceau P (1998) Implication of systemic induced resistance in the suppression of *Fusarium* wilt of tomato by *Pseudomonas fluorescens* WCS417r and nonpathogenic *Fusarium oxysporum* Fo47. Eur J Plant Pathol 104:903–910

Dumas-Gaudot E, Gollotte A, Cordier C, Gianinazzi S, Gianinazzi-Pearson V (2000) Modulation of host defence systems In: Kapulnick Y, Douds Jr DD (eds) Arbuscular mycorrhizas: physiology and functions. Kluwer, Dordrecht, pp 121–140

El Zemrany H, Cortet J, Lutz MP, Chabert A, Baudoin E, Haurat J, Maughan N, Félix D, Défago G, Bally R, Moënne-Loccoz Y (2006) Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilization. Soil Biol Biochem 38:1712–1726

Farmer E E (2007) Plant biology: jasmonate perception machines. Nature 448:659–660

Fisher PJ, Anson AE, Pertini O (1984a) Antibiotic activity of some endophytic fungi from ericaceous plants. Bot Helvet 94:249–253

Fisher PJ, Anson AE, Pertini O (1984b) Novel antibiotic activity of an endophyte *Cryptosporiopsis* sp. isolated from *Vaccinium myrtillus*. Trans Br Mycol Soc 83:145–148

Fitter AH, Garbaye J (1994) Interactions between mycorrhizal fungi and other soil organisms. Plant Soil 159:123–132

Franken P, Requena N (2001) Analysis of gene expression in arbuscular mycorrhizas: new approaches and challenges. New Phytol 150:517–523

Gange AC, Whitfield L, Ixer-Pitfield S (2004) Assessment of arbuscular mycorrhizal fungi as potential biocontrol agents for *Poa annua* L. in fine turf. J Turfgrass Sports Surface Sci 80

Garbeva P, van Veen JA, van Elsas J D (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol 42:243–270

Garcia-Garrido JM, Ocampo JA (1989) Effect of VA mycorrhizal infection of tomato on damage caused by *Pseudomonas syringae*. Soil Biol Biochem 21:165–167

Garcia-Garrido JM, Tribak M, Rejon-Palomares A, Ocampo JA, Garcia-Romera I (2000) Hydrolytic enzymes and ability of arbuscular mycorrhizal fungi to colonize roots. J Exper Bot 51:1443–1448

Garmendia I, Goicoechea N, Aguirreolea J (2005) Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against *Verticillium*-induced wilt in pepper. Mycorrhiza 15:345–356

Gasoni L, Stegman De Gurfinkel B (1997) The endophyte *Cladorrhinum foecundissimum* in cotton roots: phosphorus uptake and host growth. Mycol Res 101:867–870

Ghimire SR, Hyde KD (2004) Fungal Endophytes. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant Surface Microbiology. Springer-Verlag Berlin Heidelberg, pp 281–292

Gianinazzi-Pearson V, Dumas-Gaudot E, Gollotte A, Tahiri-Alaoui A, Gianinazzi S (1996) Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. New Phytol 133:45–57

Giovannetti M (2000) Spore germination and pre-symbiotic mycelial growth In: Kapulnick Y, Douds Jr. DD (eds) Arbuscular mycorrhizas: physiology and functions. Kluwer, Dordrecht, pp 47–68

Giri B, Giang P H, Kumari R, Prasad R, Sachdev M, Garg A P, Oelmuller R and Varma A (2004) Mycorrhizosphere: strategies and functions. In: Buscot F, Varma A (eds) Micro-organisms in soils: roles in genesis and functions. Soil biology series. Springer, Heidelberg, pp 213–252

Glandorf DC, Verheggen P, Jansen T, Jorritsma JW, Smit E, Leefang P, Wernars K, Thomashow LS, Laureijs E, Thomas-Oates JE, Bakker PA, Van Loon LC (2001) Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat. Appl Environ Microbiol 67:3371–3378

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can. J. Microbiol 41:109–117

Guenoune D, Galili S, Phillips DA, Volpin H, ChetOkon Y, Kapulnik Y (2001) The defense response elicited by the pathogen *Rhizoctonia solani* is suppressed by colonization of the AM-fungus *Glomus intraradices*. Plant Sci. 160:925–932

Haas D, Keel C, Laville J, Maurhofer M, Oberliansli T, Schnider U, Voisard C, Wüthrich B, Defago G (1991) Secondary metabolites of *Pseudomonas fluorescens* strain CHA0 involved in the suppresion of root diseases. In: Hennecke H, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions. Kluwer, Dordrecht, pp 450–456

Hameeda B, Harini G, Rupela OP, Reddy G. (2007) Effect of composts or vermicomposts on sorghum growth and mycorrhizal colonization. Afr J Biotechnol 6:009–012

Harman GE, Howell CR, Vitarbo A, Chet I, Lorito M (2004) *Trichoderma* species- opportunistic, avirulent plant symbionts. Nat Rev Microbiol 2:43–56

Harrier LA, Watson CA (2004) The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. Pest Manage Sci 60:149–157

He P, Chintamanani S, Chen Z, Zhu L, Kunkel BN, Alfano JR, Tang X, Zhou JM (2004) Activation of a COI1-dependent pathway in *Arabidopsis* by *Pseudomonas syringae* type III effectors and coronatine. Plant J 37:589–602

Hoitink HAJ, Boehm MJ (1999) Biocontrol within the context of soil microbial communities: a substrate dependent phenomenon. Annu Rev Phytopathol 37:427–446

Iavicoli A, Boutet E, Buchala A, Métraux J P (2003) Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol Plant Microb Interact 16:851–858

James HG (2001) What Do Root Pathogens See in Mycorrhizas? New Phytol 149:357–359

Jordaan A, Taylor J E, Rossenkhan R (2006) Occurrence and possible role of endophytic fungi associated with seed pods of *Colophospermum mopane* (Fabaceae)in Botswana. (Published online) http://www.sciencedirect.com/science/journal/02546299

Kimmons CA (1990) Nematode reproduction on endophyte infected and endophyte free tall fescue. Plant Dis 74:757–761

Kiss L (2003) A review of fungal antagonists of powdery mildews and their potential as biocontrol agents. Pest Manage Sci 59:475–483

Kloepper JW (1994) Plant growth-promoting rhizobacteria (other systems) In: Okon Y (ed) *Azospirillum*/plant associations. CRC Press, Boca Raton, pp 111–118

Kulik MM (1988). Observations by scanning electron and brightfield microscopy on the mode of penetration of soybean seedlings by *Phomopsis phaseoli*. Plant Dis 72:115–118

Kuster H, Becker A, Firnhaber C, Hohnjec N, Manthey K, Perlick A M, Bekel T, Dondrup M, Henckel K, Goesmann A, Meyer F, Wipf D, Requena N, Hildebrandt U, Hampp R, Nehls U, Krajinski F, Franken P, Puhler A (2007) Development of bioinformatic tools to support EST-sequencing, in silico- and microarray-based transcriptome profiling in mycorrhizal symbioses. Phytochemistry 38:19–32

Lacey LA, Frutos R, Kaya HK,Vail P (2001) Insect pathogens as biological control agents: do they have a future? Biol Control 21:230–248

Lambrecht M, Okon Y, Vande Broek A, Vanderleyden J (2000) Indole-3-acetic acid, a reciprocal signalling molecule in bacteria-plant interactions. Trends Microbiol 8:298–300

Lemanceau P, Alabouvette C (1993). Suppression of *Fusarium*-wilts by fluorescent pseudomonads: mechanisms and applications. Biocontrol Sci Technol 3:219–234

Lewis DH (1973) Concept in fungal nutrition and the origin of biotrophy. Biol Rev 48:261–278

Li HY, Yang GD, Shu HR, Yang YT, Ye BX, Nishida I, Zheng CC (2006) Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the

root-knot nematode Meloidogyne incognita in the grapevine (*Vitis amurensis Rupr*.), which includes transcriptional activation of the class III chitinase gene VCH3. Plant Cell Physiol 47:154–63

Linderman RG (1994) Role of VAM fungi in biocontrol. In: Pfleger FL, Linderman RG (eds) Mycorrhizae and plant health. APS Press, St Paul, pp 1–26

Linderman RG (2000) Effects of mycorrhizas on plant tolerance to diseases.In: Kapulnik Y, Douds DD Jr. (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 345–365

Marschner P, Rengel Z (2007) Nutrient Cycling in Terrestrial Ecosystems. Springer Verlag Germany

McKellar ME, Nelson EB (2003) Compost-induced suppression of *Pythium* damping-off is mediated by fatty-acid-metabolizing seed-colonizing microbial communities. Appl Environ Microbiol 69:452–460

Miller JD (1986). Toxic metabolites of epiphytic and endophytic fungi of conifers needles. In: Fokkema NJ, Heuvel JVD (eds) Microbiology of phyllosphere. Cambridge University Press, Cambridge, pp 223–231

Miller RM, Jastrow JD (2000) Mycorrhizal fungi influence soil structure. In: Kapulnik Y, Douds DD Jr. (eds) Arbuscular mycorrhizas: physiology and functions. Kluwer, Dordrecht, pp 3–18

Morris PF, Ward EWR (1992) Chemoattraction of zoospores of the plant soybean pathogen, *Phytophthora sojae*, by isoflavones. Physiol Mol Plant Pathol 40:17–22

Morton JB, Benny GL (1990) Revised classification of arbuscular mycorrhizal fungi (zygomycetes): a new order glomales, two new suborders, glomineae and gigasporineae and gigasporaceae, with an amendation of glomaceae. Mycotaxon 37:471–491

Morton JB, Redecker D (2001) Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera Archaeospora and Paraglomus, based on concordant molecular and morphological characters. Mycologia 93:181–195

Mukerji KG, Ciancio A (2007) Mycorrhizae in the integrated pest and disease management. In: Ciancio A, Mukerji KG (eds) General concepts in integrated pest and disease management, Springer, Heidelberg, pp 245–266

Mukerji KG, Mandeep and Varma A (1998) Mycorrhizosphere microrganisms: screening and evaluation. In: Varma A (ed) Mycorrhiza manual. Springer, Heideberg, pp 85–98

Numberger T, Brunner F, Kemmerling B and Piater L (2004) Innate immunity in plants and animals:striking similarities and obvious differences. Immunol Rev 198:249–266

O'Donnell J, Dickinson CH (1980) Pathogenicity of *Alternaria* and *Cladosporium* isolated on *Phaseolus*. Trans Br Mycol Soc 74:335–342

Ocón A, Hampp R and Requena N (2007) Trehalose turnover during abiotic stress in arbuscular mycorrhizal fungi. New Phytol 174:879–891

Odum EP (1953) Fundamentals of ecology. Saunders, Philadelphia

Ongena M, Duby F, Rossignol F, Fouconnier ML, Dommes J and Thonart P (2004) Stimulation of the lipoxygenase pathway is associated with systemic resistance induced in bean by a nonpathogenic *Pseudomonas* strain. Mol. Plant Microb Interact 17:1009–1018

Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in the biocontrol of *Sclerotium rolfsii*. Phytopathology 78:84–88

Pal KK, B McSpadden Gardener (2006) Biological control of plant pathogens. The plant health instructor DOI: 10.1094/PHI-A-2006–1117–02

Palumbo JD, Yuen GY, Jochum CC, Tatum K, and Kobayashi DY (2005) Mutagenesis of beta-1,3-glucanase genes in *Lysobacter enzymogenes* strain C3 results in reduced biological control activity toward Bipolaris leaf spot of tall fescue and *Pythium* damping-off of sugar beet. Phytopathology 95:701–707

Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737

Petrini O (1991) Fungal. endophytes of tree leaves. In: Andrews J, Hirano S (eds) Microbila ecology of leaves. Springer, Heidelberg, pp 179–197

Petrini O (1996) Ecological and physiological aspect of host specificity in endophytic fungi. In: Redlin SC, Carris LM (eds) Endophytic fungi in grasses and woody plants. APS Press, St Paul, Minn

Petrini O, Fisher PJ, Petrini LE (1992) Fungal endophytes of bracken (*Pteridium aquilinum*) with some reflections on their use in biological control. Sydowia 44:282–293

Probanza A, Lucas García JA, Ruiz Palomino M, Ramos B, Gutiérrez Mañero FJ (2002) *Pinus pinea* L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus (B. licheniformis* CECT 5106 and *B. pumillus* CECT 5105). Appl Soil Ecol 20:75–84

Rabie GH, Almadini AM (2005) Role of bioinoculants in development of salt-tolerance of *Vicia faba* plants under salinity stress. Afr J Biotechnol 4 :210–222

Ramarathnam R, Dilantha FWG (2006) Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans*, the blackleg pathogen of canola. Biocontrol Sci Technol 16:567–582

Redecker D, Morton JB, Bruns TD (2000) Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). Mol Phylogenet Evol 14:276–284

Requena N, Perez-Solis E, Azcón-Aguilar C, Jeffries P, Barea JM (2001) Management of indigenous plant-microbe symbioses aids restoration of desertified. Appl Environ Microbiol 67:495–498

Requena N, Serrano E, Ocon A, Breuninger M (2007) Plant signals and fungal perception during arbuscular mycorrhiza establishment. Phytochemistry 68:33–40

Ross EW, Marx DM (1972) Susceptibility sand pine to *Phytophthora cinnamomi*. Phytopathology 62:1197–1200

Sanders IR, Clapp JP, Wiemken A (1996) The genetic diversity of arbuscular mycorrhizal fungi in natural ecosystems - a key to understanding the ecology and functioning of the mycorrhizal symbiosis. New Phytol 133:123–134

Scheffknecht S, Mammerler R, Steinkellner S, Vierheilig H (2006) Root exudates of mycorrhizal tomato plants exhibit a different effect on microconidia germination of *Fusarium oxysporum* f. sp. lycopersici than root exudates from non-mycorrhizal tomato plants. Mycorrhiza 16:365–70

Schulz B, Guske S, Dammann U, Boyle C (1998) Endophyte-host interactions.II. Defining symbiosis of the endophyte-host interaction. Symbiosis 25:213–227

Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the *Glomeromycota*: phylogeny and evolution. Mycol Res 105:1421–1423

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field Conditions. Phytopathology 97:523–531

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton-X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmuller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and Arabidopsis roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J Biol Chem 280:26241–26247

Shoresh M, Yedidia I, Chet I (2005) Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. Phytopathology 95:76–84

Siddiqui Z, Akhtar M (2006) Biological control of root-rot disease complex of chickpea by AM fungi. Arch Phytopathol Plant Prot 39:389–395

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Stone JK (1988) Fine structure of latent infection by *Rhabdocline parkeri* on Douglas fir, with observation on uninfected epidermal cells. Can J Bot 66:45–54

Suske J, Acker G (1989) Identification of endophytic hyphae of *Lophodermium piceae* in tissues of green, symptomless Norway spruce needles by immunoelectorn microscopy. Can J Bot 67:1768–1774

Tejesvi MV, Mahesh B, Nalini MS, Prakash HS, Kini KR, Subbiah V, Shetty HS (2006) Fungalendophyte assemblages from ethnopharmaceutically important medicinal trees. Can J Microbiol 52:427–35

Thygesen K, Larsen J, Bødker L (2004) Arbuscular Mycorrhizal Fungi Reduce Development of Pea Root-rot caused by *Aphanomyces euteiches* using Oospores as Pathogen Inoculum. Eur J Plant Pathol 110:411–419

Van de Broek A, Lambrecht M, Vanderleyden J (1999) Auxins upregulate expression of the indole-3-pyruvate decaboxylase gene from *Azospirillum brasilense*. J Bacteriol 181:1338–1342

Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Varma A, Chincholkar S (eds) (2007) Microbial siderophores, Springer, Heidelberg

Varma A, Verma S, Sudha, Sahay N, Bütehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. Appl Environ Microbiol. 65:2741–2744

Vierheilig H, Piché Y (2002) Signalling in arbuscular mycorrhiza: facts and hypotheses In: Buslig B, Manthey J (eds) Flavonoids in cell functions. Kluwer, New York, pp 23–29

Walker C, Schüßler A (2004) Nomenclatural clarifications and new taxa in the Glomeromycota. Mycol Res 108:981–982

Waller W, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt stress tolerance, disease resistance and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Weller DM, Raaijmakers J, McSpadden Gardener B, Thomashow LM (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40:309–348

Wilson D (1995) Endophyte - the evolution of term, a classification of its use and definition. Oikos 73:274–276

Ziedan EHE (2006) Manipulating endophytic bacteria for biological control to soil borne diseases of peanut. J Appl Sci Res 2:497–502

The Biocontrol Effect of Mycorrhization on Soilborne Fungal Pathogens and the Autoregulation of the AM Symbiosis: One Mechanism, Two Effects?

H. Vierheilig(✉), S. Steinkellner, T. Khaosaad, and J.M. Garcia-Garrido

1 Introduction

The establishment of the AM in the roots of more than 80% of all land plants is the result of a complex exchange of signals between the host plant and AMF. Many reports are available that once the AMF has penetrated the host root and established its interradical organs of nutrient exchange between the AMF and the plant, a number of physiological and morphological changes occur in the host plant.

The importance of most of these changes is still unclear, although the most prominent results of mycorrhization are, among others, an improved nutrient status of the host plant (Smith and Read 1997) and a bioprotective effect of mycorrhization against soilborne fungal pathogens (Singh et al. 2000; Azcón-Aguilar et al. 2002; Xavier and Boyetchko 2004; St-Arnaud and Elsen 2005; St-Arnaud and Vujanovic 2007).

In the legume–*Rhizobium* interaction, once a plant has formed nodules, further nodulation is suppressed in other parts of the root system by a long-distance signal exchange, which means that the nodulation is autoregulated (see reviews in Oka-Kira and Kawaguchi 2006; Kinkema et al. 2006).

In recent years, it has been reported that once plants are colonized by AMF, further root colonization by AMF is regulated (reviewed by Vierheilig 2004a,b). In analogy to the rhizobial autoregulatory mechanism in legume plants, this phenomenon with AMF has been named "autoregulation of mycorrhization". Recently, it has been suggested that the bioprotective effect of mycorrhization and the autoregulation of mycorrhization are possibly two sides of the same coin. It seems plausible that an already mycorrhizal plant develops just one mechanism to repulse further colonization by fungi, not discriminating between AMF and soilborne pathogenic fungi (Vierheilig and Piché 2002; Vierheilig 2004a,b).

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In this chapter, we want to discuss the data accumulated on the mycorrhizal autoregulation and on the bioprotective effect of mycorrhization in order to highlight possible similarities between both processes

2 The Biocontrol Effect of Mycorrhization on Soilborne Fungal Pathogens

Most data about bioprotection of mycorrhization are available for soilborne fungal pathogens. Numerous studies show a clear localized protective effect (reviewed by Singh et al. 2000; Azcón-Aguilar et al. 2002; Xavier and Boyetchko 2004; St-Arnaud and Vujanovic 2007) while recently a systemic protective effect with different soilborne fungal pathogens has also been reported (Cordier et al. 1998a; Pozo et al. 2002; Khaosaad et al. 2007).

In this review, we want to give a short overview about the different aspects involved in mycorrhizal biocontrol. More details can be obtained from several excellent recent reviews on this subject (Whipps 2004; Xavier and Boyetchko 2004; Azcon-Aguilar et al. 2002; Singh et al. 2000), but we will provide some new data relevant to the field.

2.1 Factors Affecting Bioprotection Through Mycorrhization

Apart from abiotic factors affecting the biocontrol efficacy of the AMF, such as temperature, soil moisture and soil P-content (for details see Singh et al. 2000), the bioprotective effect against soilborne fungal pathogens seems to depend on several biotic factors such as the host genotype, the AMF isolate and the degree of mycorrhization.

2.1.1 The Host Genotype

In a number of studies, it has been demonstrated that depending on the host genotype the degree of AM root colonization and the plant growth effect of mycorrhization can vary, and it has been suggested that the AM development and its effect on the host plant are at least partially under the genetic control of the host (Lackie et al. 1987; Hetrick et al. 1993; Vierheilig and Ocampo 1990, 1991). The host genome also seems to affect the protective effect provided by the AMF, as the host genotype seems to result in a differing bioprotective response by the mycorrhizal association. Depending on the genotype, mycorrhizal strawberry showed a different susceptibility to *Phytophthora fragariae* (Mark and Cassels 1996).

2.1.2 The AMF

In an overview on studies on the bioprotective effect of mycorrhization listing the host plant, the pathogen, the AMF, and the effect of mycorrhization (Singh et al. 2000; Whipps 2004), we can see that more than 80% of the studies on the bioprotective effect of mycorrhization have been performed with the genus *Glomus*, whereas only about 14% were performed with the genus *Gigaspora,* while there are almost no studies available using other AM genera. Moreover, within the genus *Glomus,* nearly half of the studies were performed with *G. mosseae* (25%) and *G. fasciculatum* (23%) and 8% each with *G. etunicatum* and *G. intraradices*. This means that data on a bioprotective effect of mycorrhization originate mostly from the genus *Glomus,* and within this genus from two species.

From a few studies comparing the bioprotective effect of AMF species, we can conclude that depending on the AMF species the bioprotective effect differs, e.g., *Glomus intraradices* has been reported not to protect clover against nematode infection (Habte et al. 1999) and was not effective in reducing disease symptoms produced by *Phytophthora parasitica* infection in tomato (Pozo et al. 2002). In contrast, root colonization by *G. mosseae* resulted in a clear protective effect against nematodes and *P. parasitica* infection (Habte et al. 1999; Pozo et al. 2002).

As some AMF exhibit a clear bioprotective effect, whereas other AMF do not affect the plant–pathogen interaction in terms of bioprotection, more comparative studies are definitely needed involving a greater variety of AMF.

2.1.3 The Degree of Mycorrhization

After plant inoculation with an AMF, the first signs of root colonization are visible after a few days. Thereafter, AM root colonization can increase drastically until it reaches a final plateau (e.g., for tomato and soybean around 60%; Vierheilig et al. 1994; Wyss et al. 1991). In several studies, a local bioprotective effect has been linked with a high degree of AM root colonization, whereas intermediate and low levels of AM root colonization showed no bioprotective effect. Apparently a critical level of AM root colonization is needed to provide bioprotection for mycorrhizal plants. In mycorrhizal tomato plants a bioprotective effect against *P. parasitica* (Cordier et al. 1998b) and *Fusarium oxysporum* (Caron et al. 1986a,b), and in wheat plants against *Gaeumannomyces graminis* (Graham and Menge 1982), could only be observed when roots were heavily colonized by the AMF; low mycorrhization levels resulted in no bioprotection.

In a recent report it has been demonstrated that not only the local, but also the systemic, bioprotective effect of mycorrhization depends on the degree of AM root colonization (Khaosaad et al. 2007). Moreover, the critical level of AM root colonization in barley to provide local and systemic bioprotection against *G. graminis* seems to be similar (Fig. 1; Khaosaad et al. 2007).

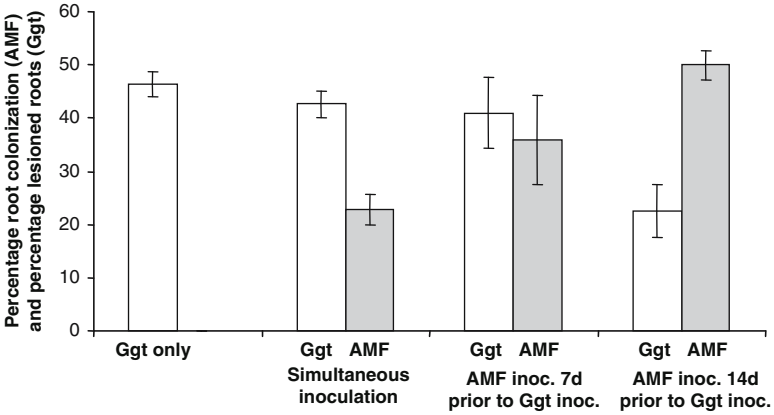

Fig. 1 The local effect of sequential inoculation by the AMF *G. mosseae* on severity of take-all disease caused by *G. graminis* (Ggt) was studied. In the compartment system described by Vierheilig et al. (2000a), barley plants were sequentially inoculated with the AMF *G. mosseae*. This resulted in a differing AM root colonization at the end of the experiment. When plants were inoculated with Ggt, AM root colonization ranged from 0% (simultaneous inoculation), 10 ± 1% (AMF inoculation 7 days prior to Ggt inoculation) to 18 ± 2% (AMF inoculation 14 days prior to Ggt inoculation). At the time of harvest (14 days after Ggt inoculation), roots were scored for root browning (for more details, see Khaosaad et al. 2007). Data represent mean ± SE ($n = 5$)

The sequence of inoculation has also been suggested as an important factor in mycorrhizal bioprotection. In general, it has been postulated that the inoculation has to be prior to inoculation with the soilborne pathogen (Singh et al. 2000; Azcón-Aguilar et al. 2002; Xavier and Boyetchko 2004). However, this aspect is probably closely linked with the degree of mycorrhization. The earlier the AMF colonizes the root, the higher the mycorrhization level will be before a pathogen infection.

2.2 Mechanisms of Mycorrhization-mediated Bioprotection

A number of mechanisms have been suggested to be involved in the bioprotective effect of mycorrhization against soilborne fungal pathogens, but hard data are not yet available for all of them. Classically, four major groups of mycorrhizal mode of action mechanisms that mediated bioprotection have been considered: (1) direct competition, (2) mechanism mediated by alteration in plant growth, nutrition and morphology, (3) biochemical and molecular changes in mycorrhizal plants that induce pathogen resistance, and (4) alterations in the soil microbiota and development of pathogen antagonism. Although some of the suggested mechanisms might play no role, it is generally agreed that bioprotection through

mycorrhization is the result of a combination of several of mechanisms and not of a single mechanism.

2.2.1 Direct Competition

2.2.1.1 Competition for Infection Sites

A competition effect at the infectional level has also been suggested. The AMF might occupy infection sites on the root surface needed by the pathogen to penetrate the root, or cells in the root already occupied by the AMF cannot be colonized any further by the pathogen (Cordier et al. 1998a,b).

2.2.1.2 Carbon Competition

Not only the growth of symbiotic AMF but also of pathogenic fungi depends on carbon from photosynthesis. Thus, it has been suggested that the carbon availability in mycorrhizal plants could explain the biocontrol effect of mycorrhization. Once a plant is colonized by AMF, due to the carbohydrates used by the symbiotic AM fungus, less carbon could be available for a root colonizing fungal pathogen (Singh et al. 2000; Azcón-Aguilar et al. 2002; Xavier and Boyetchko 2004).

Different AMF have been reported to exhibit a different carbon sink strength in mycorrhizal roots (Lerat et al. 2003a, 2003b) and, thus, should exhibit a different biocontrol effect. Interestingly, in a split-root system of tomato with one side colonized by the AMF *G. mosseae* (BEG 12), disease symptoms produced by *P. parasitica* on the other side of the split-root system were reduced (Cordier et al. 1998a; Pozo et al. 2002). This would point towards a certain carbon sink strength of the AMF *G. mosseae* (BEG 12). However, *G. mosseae* (BEG 12) has been shown in different plant systems not to exhibit any carbon sink strength. Moreover, despite its high carbon sink strength the AMF *G. intraradices* did not provide bioprotection against *P. parasitica* (Pozo et al. 2002; Lerat et al. 2003a).

From these data, it seems that carbon competition between AMF and a pathogen can be discarded as a factor involved in mycorrhizal bioprotection.

2.2.1.3 Direct Competition/Inhibition in Soil

Some experimental evidence has been reported about possible mechanisms of direct action of AM fungi against pathogens in soil (St-Arnaud et al. 1995; Filion et al. 1999; García-Garrido and Ocampo 1989). Nevertheless, at the present time, the production of antibiotics or inhibitory compounds by AM fungi has not been proven.

2.2.2 Mechanism Mediated by Alteration in Plant Growth, Nutrition and Morphology

2.2.2.1 Improved Nutrient Status/Root Damage Compensation

It has been suggested that the improved nutrient status of mycorrhizal plants makes them more tolerant to damage caused by pathogens and carbon drain from the plant to the pathogen. However, there is strong evidence that the nutritional effect of the AM symbiosis is only one among several aspects of the mycorrhizal effect on pathogens (Trotta et al. 1996). Moreover, it has been suggested that the nutrient uptake by the fine extraradical mycelium of the AMF could compensate for a pathogen-reduced root system (Singh et al. 2000).

2.2.2.2 Morphological Alterations of the Root

Due to mycorrhization, the morphology of the root changes (Berta et al. 1995; Copetta et al. 2006), but no clear correlation with a bioprotective effect of mycorrhization has yet been found. In basil, the root fresh weight, the total root length, the number of root tips and the degree of branching was altered differently depending on the root-colonizing AMF (Copetta et al. 2006). Linking these alterations of the root parameters with data on a bioprotective effect of the different AMF could show whether morphological alterations of the root due to mycorrhization are really involved in the bioprotective effect of mycorrhization.

2.2.3 Biochemical and Molecular Changes in Mycorrhizal Plants that Induce Resistance to Pathogens

Several physiological and biochemical alterations of the host after mycorrhization have been reported. Some are possibly linked with a protective effect of the mycorrhizal plant against pathogens, e.g. the induction of hydrolytic enzymes (Pozo et al. 1999), enhanced levels of PR proteins, the accumulation of phytoalexins (Harrison and Dixon 1993; Morandi 1996; Larose et al. 2002) and callose (Cordier et al. 1998b), the accumulation of salicylic acid (Blilou et al. 2000a, 2000b; Medina et al. 2003) and reactive oxygen species (Salzer et al. 1999). During AM development, there is evidence that these defensive responses occur (García-Garrido and Ocampo 2002) and that they are strongly stimulated when a subsequent challenge with a pathogen takes place. Possibly the mechanisms of plant defence are activated faster and to a greater extent in mycorrhizal plants when challenged by a pathogen compared to nonmycorrhizal plants, and it has been suggested that AM colonization acts as a priming system for the process of pathogen resistance (Azcón-Aguilar et al. 2002; Pozo and Azcon-Aguilar 2007). In this respect, elevated JA levels occurring upon mycorrhization, likely associated with a fully established mycorrhiza, may mediate the enhance defence status of the mycorrhizal plant (Vierheilig and Piché 2002; Hause et al. 2007; Vierheilig 2004a).

However, the importance of each component of the plant resistance response in the bioprotective effect of AM against soil pathogens and the signaling pathway that control these responses are unknown.

As the whole metabolism of the plant is altered by mycorrhization, alterations of the root exudation pattern are no surprise. These alterations could act on the pathogen indirectly, through an altered pH in the rhizosphere and/or directly through an altered composition of the exudates with reduced levels of stimulatory compounds and/or the presence of inhibitory compounds. Changes of the pH in the rhizosphere of the mycorrhizal plant (Bago et al. 1996; Villegas et al. 1996) have been reported before, however, no data are availble yet how these pH changes of the rhizosphere affect root pathogens.

There are a number of reports on root exudates and AMF (see recent reviews by Jones et al. 2004; Nagahashi and Douds 2005; Vierheilig and Bago 2005), and more and more data are accumulated that exudates of mycorrhizal plants affect bacteria (Sood 2003), fungi (Norman and Hooker 2000; Lioussanne et al. 2003; Scheffknecht et al. 2006, 2007) and nematodes (Ryan and Jones 2004) differently then exudates from nonmycorrhizal plants.

3 The Mycorrhizal Autoregulation

In recent years, more and more data have been accumulated that once plants are colonized by AMF, in order to limit the energy costs of the AM symbiosis further root colonization by AMF is suppressed. This mechanism has been named "autoregulation of mycorrhization" (Vierheilig 2004a; Garcia-Garrido and Vierheilig 2007).

Due to the fact that in roots a recent AM root colonization cannot be distinguished from a prior AM root colonization, and that in roots it is extremely difficult to distinguish one AMF from another, split-root systems have been used to study the mycorrhizal autoregulation. Split-root systems of plants were inoculated on one side with an AMF and, when the symbiosis was well established, the other side was inoculated with the same or another AMF. In these experiments with different AM host plants, such as barley (Vierheilig et al. 2000a, 2000b), alfalfa (Catford et al. 2003, 2006) and soybean (Meixner et al. 2005, 2007), it could be clearly shown that AM precolonization on one side of a split-root system systemically suppresses AM root colonization on the other side of the root system. The mechanisms which are actually controlling this autoregulatory effect are still unknown.

3.1 Carbon Competition

Carbon availability in mycorrhizal plants could explain the reduced root colonization in already mycorrhizal plants. Once one side of a split-root system is colonized by AMF, due to the carbohydrates used by the symbiotic AM fungus, less carbon

could be available for a later root colonizing AMF on the other side of the split-root system.

Testing the carbon sink strength of several AMF in split-root systems of barley and sugar maple, it could be demonstrated that different AMF exhibit a different carbon sink strength in mycorrhizal roots (Lerat et al. 2003a; b). The *G. mosseae* strain BEG 12 for example showed no carbon sink strength, whereas *G. intraradices* and *Gigaspora rosea* were strong carbon sinks. With carbon availability as the controlling factor this would mean that with *G. mosseae* there is no or only a weak autoregulatory effect, whereas with the two other fungi mycorrhization is clearly autoregulated.

Interestingly the degree of autoregulation was similar with *G. mosseae*, *G. intraradices* and *Gigaspora* (Vierheilig et al. 2000b), thus discarding the competition for carbon as the regulatory factor of mycorrhizal autoregulation.

3.2 Regulation by P-Levels

An improved P-status has been suggested as another possible factor affecting further AM root colonization of already mycorrhizal plants (Pearson et al. 1993). Mycorrhization could increase the P-level of the plant and thus suppress further root colonization by AMF. An experiment with a split-root system of barley with one side mycorrhizal showed that on the non-mycorrhizal side of the split-root system P-levels were similar as in the split-root system of the non-mycorrhizal control plant.

However, in the pre-colonized root system further root colonization was suppressed, whereas without pre-colonization no suppression of root colonization could be observed (Vierheilig et al. 2000b). In the experimental system studying mycorrhizal autoregulation, this excludes P as a potential suppressive factor of further root colonization in mycorrhizal plants. Moreover, in a P-application experiment it could be demonstrated that in split-root systems the improved P-status and the observed suppressive effect following mycorrhization can not be linked (Vierheilig et al. 2000b).

4 The Mycorrhizal Biocontrol Effect and the Mycorrhizal Autoregulation: One Mechanism, Two Effects?

Above we describe characteristics of the biocontrol effect of mycorrhization and of the mycorrhizal autoregulation. Once a plant is mycorrhizal it seems to control further root colonization by AMF and pathogenic fungi. Although compared to mycorrhizal biocontrol, only a few data are available on mycorrhizal autoregulation, both effects seem to share certain similarities. Below we want discuss these similarities.

4.1 Systemic Effects

A systemic effect of mycorrhization has been reported from mycorrhizal bioprotection and mycorrhizal autoregulation. Both systemic effects were studied in split-root systems.

Precolonization of one side of a split-root systems resulted in a clear suppression of different soil-borne fungal pathogens on the other side of the split-root system (Cordier et al. 1998a; Pozo et al. 2002; Khaosaad et al. 2007).

A similar systemic suppressive effect on AM root colonization was observed. When the first side of a split-root system was colonized by different AMF, AM root colonization on the other side was drastically decreased. Further studies are needed to verify whether the two systemic phenomena share the same mechanism.

4.2 The Degree of Mycorrhization

In several studies it has been reported that a local bioprotective effect depends on the degree of AM root colonization (Graham and Menge 1982; Caron et al. 1986; Cordier et al. 1998b).

In analogy to a local bioprotective effect by mycorrhization, the systemic bioprotection also seems to depend on a critical level of AM root colonization. This has been reported recently in a study with mycorrhizal barley plants and the soil-borne pathogen *G. graminis*. Once one side of a split-root system of barley was extensively colonized by the AMF *G. mosseae*, the damage caused by *G. graminis* was clearly reduced on the other side, whereas low levels of AM root colonization on the first side of the split-root system resulted in no protective effect (Khaosaad et al. 2007).

Interestingly, in the same test plant, barley, a similar pattern has been observed for the mycorrhizal autoregulation. Only high levels of AM root colonization on the first side of a split-root system resulted in a significant suppression of further AM root colonization on the other side (Vierheilig 2004b). These data indicate that both phenomena depend on a critical degree of root colonization by AMF.

4.3 Alterations of the Root Exudation Pattern

Alterations of the root exudation pattern have been suggested to be involved in the expression of the mycorrhizal bioprotective effect and these changes have been suggested to be at least partially involved in the altered susceptibility of mycorrhizal plants towards soil-borne microorganisms (Vierheilig and Piché 2002; Vierheilig 2004a). In *in vitro* studies root exudates from mycorrhizal strawberry plants reduced the sporulation of *P. fragariae* (Norman and Hooker 2000) and root

exudates from mycorrhizal potato plants increased hatching of nematodes (Ryan and Jones 2004). Looking at the chemotactic response root exudates collected from non-mycorrhizal tomato roots exhibited a higher attracting effect on zoospores of *P. parasitica* (Lioussanne et al. 2003), whereas in the case of the two plant-growth-promoting bacteria *A. chroococum* and *P. fluorescens*, root exudates from mycorrhizal tomato plants showed a higher attractional effect (Sood 2003). Most recently it was reported that root exudates from mycorrhizal plants show an altered effect on microconidia germination of *F. oxysporum* compared to root exudates from non-mycorrhizal plants (Scheffknecht et al. 2006, 2007).

Interestingly the altered exudation pattern of mycorrhizal plants is not only affecting pathogenic and non-pathogenic soil-organisms but also the AMF itself. Root exudates from mycorrhizal cucumber plants lost their stimulating effect on the hyphal growth of AMF. Moreover, when added to inoculated cucumber plants root exudates from mycorrhizal cucumber plants suppressed AM root colonization (Pinior et al. 1999). This suppressive effect on root colonization was systemic, that means even root exudates from the non-mycorrhizal side of a split-root system of a mycorrhizal plant affected the AM root colonization negatively (Vierheilig et al. 2003). Systemic alterations of root exudates through mycorrhization affecting the soil-borne pathogen *F. oxysporum* have been reported recently (Scheffknecht et al. 2006).

These data show clearly that alterations of the root exudation pattern through mycorrhization do not only affect other soil-borne pathogenic and non-pathogenic organisms, but also the AMF in the soil.

5 Conclusions

To summarize, the systemic effect of mycorrhizal biocontrol and mycorrhizal autoregulation, their regulation by the degree of AM root colonization and the alterations by the root exudation pattern do point towards “one mechanism, two effects”.

In 2002 Vierheilig and Piché suggested that plants colonized by AMF while trying to limit their costs of the AM symbiosis by mycorrhizal autoregulation might have acquired bioprotection against pathogenic fungi. It seems plausible that an already mycorrhizal plant develops only one mechanism to repulse colonization by fungi, not discriminating between AM fungi and soil borne pathogenic fungi.

Further studies, including tanscriptomic and metabolomic analysis, are needed to study the hypothesis of “one mechanism, two effects” and to elucidate which mechanisms and signalling molecules are decisive for the regulation and functioning of the two effects.

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References

Azcón-Aguilar C, Jaizme-Vega MC, Calvet C (2002) The contribution of arbuscular mycorrhizal fungi to the control of soil-borne plant pathogens. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Switzerland, pp 187–197

Bago B, Vierheilig H, Piché Y, Azcon C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Berta G, Trotta A, Fusconi A, Hooker JE, Munro M, Atkinson D, Giovannetti M, Morini S, Fortuna P, Tisserant B, Gianinazzi-Pearson V, Gianinazzi S (1995) Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. Tree Physiol 15:281–293

Blilou I, Bueno P, Ocampo JA, García-Garrido JM (2000a) Induction of catalase and ascorbate peroxidase activities in tobaccco roots inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae*. Mycol Res 104:722–725.

Blilou I, Ocampo JA, García-Garrido JM. 2000b. Induction of *Ltp* (Lipid transfer protein) and *Pal* (Phenylalanine ammonia-lyase) gene expression in rice roots colonized by the arbuscular mycorrhizal fungus *Glomus mosseae*. J Exp Bot 51:1969–1977

Caron M, Fortin JA, Richard C (1986a) Effect of inoculation sequence on the interaction between *Glomus intraradices* and *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomatoes. Can J Plant Pathol 8:12–16

Caron M, Fortin JA, Richard C (1986b) Effect of phosphorus concentration and *Glomus intraradices* on *Fusarium* crown and root rot of tomatoes. Phytopathology 76:942–946

Catford JG, Staehelin C, Lerat S, Piché Y, Vierheilig H (2003) Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. J Exp Bot 54:1481–1487

Catford JG, Staehelin C, Larose G, Piché Y, Vierheilig H (2006) Systemically suppressed isoflavonoids and their stimulating effects on nodulation and mycorrhization in alfalfa split-root systems. Plant Soil 285:257–266

Copetta A, Lingua G, Berta G (2006) Effects of three AM fungi on growth, distribution of glandular hair, and of essential oil production in *Ocimum basilicum* L. var. Genovese. Mycorrhiza 16:485–494

Cordier C, Gianinazzi S, Gianinazzi-Pearson V (1998a) Colonization pattern of root tissue by *Phytophthora nicotianae* var. *parasitica* related to reduced disease in mycorrhizal tomato Plant Soil 185:223–232

Cordier C, Pozo M J, Barea JM, Gianinazzi S, Gianinazzi-Pearson V (1998b) Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus Mol Plant Microb Interact 11:1017–1028

Filion M, St-Arnaud M, Fortin JA (1999) Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. New Phytol 141:525–533

García-Garrido JM, Ocampo JA (1989) Effect of VA mycorrhizal infection of tomato on damage caused by *Pseudomonas syringae*. Soil Biol Biochem 21:65–167

García-Garrido JM, Ocampo JA (2002) Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. J Exp Bot 53:1377–1386

Garcia Garrido JM, Vierheilig H (2007) From a germinating spore to an established arbuscular mycorrhiza: signalling and regulation. In: Khasa D, Piché Y, Coughlan A (eds) Advances in mycorrhizal biotechnology: a Canadian perspective. NRC Research Press, Ottawa (in press)

Graham JH, Menge JA (1982) Influence of vesicular-arbuscular mycorrhizae and soil phosphorous on take-all disease of wheat. Phytopathology 72:95–98

Habte M, Zhang YC, Schmitt DP (1999) Effectiveness of *Glomus* species in protecting white clover against nematode damage. Can J Bot 77:135–139

Harrison M, Dixon R (1993) Isoflavonoid accumulation and expression of defense gene transcripts during the establishment of vesicular arbuscular mycorrhizal associations in roots of *Medicago truncatula*. Mol Plant-Microbe Interact 6:643–659

Hause B, Cornelia M, Stanislav I, Strack D (2007) Jasmonate in arbuscular mycorrhizal interactions. Phytochemistry 68:101–110.

Hetrick BAD, Wilson GWT, Cox TS (1993) Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. Can J Bot 71:512–518

Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. New Phytol 163:459–480

Khaosaad T, Garcia-Garrido JM, Steinkellner S, Vierheilig H (2007) Take-all disease is systemically reduced in roots of mycorrhizal barley plants. Soil Biol Biochem 39:727–734

Kinkema M, Scott PT, Gresshoff PM (2006) Legume nodulation: successful symbiosis through short- and long-distance signalling. Funct Plant Biol 33:707–721

Lackie SM, Garriock ML, Peterson RL, Bowley SR (1987) Influence of host plant on the morphology of the vesicular-arbuscular mycorrhizal fungus *Glomus versiforme* (Daniels and Trappe) Berch. Symbiosis 3:147–158

Larose G, Chenevert, Moutoglis P, Gagne S, Piché, Vierheilig H (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. J Plant Physiol 159:1329–1339

Lerat S, Lapointe L, Gutjahr S, Piché Y, Vierheilig H (2003a) Carbon partitioning in a split-root system of arbuscular mycorrhizal plants is fungal and plant species dependent. New Phytol 157:589–595

Lerat S, Lapointe L, Piché Y,Vierheilig H (2003b) Variable carbon sink strength of different *Glomus mosseae* strains colonizing barley roots. Can J Bot 81:886–889

Lioussanne L, Jolicoeur M, St. Arnaud M (2003) Effects of the alteration of tomato root exudation by *Glomus intraradices* colonization on *Phytophthora parasitica* var. Nicotianae zoospores. Abstract No. 253, Abstract Book ICOM 4; Montreal/ Canada

Mark GL, Cassells AC (1996) Genotype-dependence in the interaction between *Glomus fistulosum*, *Phytophthora fragariae* and the wild strawberry (*Fragaria vesca*). Plant Soil 185:233–239

Medina HMJ, Gagnon H, Piché Y, Ocampo JA, García Garrido JM, Vierheilig H (2003) Root colonization by arbuscular mycorrhizal fungi is affected by the salicylic acid content of the plant. Plant Sci 164:993–998

Meixner C, Ludwig-Müller J, Miersch O, Gresshoff P, Staehelin C, Vierheilig H (2005) Lack of mycorrhizal autoregulation and phytohormonal changes in the supernodulating soybean mutant *nts1007*. Planta 222:709–715

Meixner C, Vegvari G, Ludwig-Müller J, Gagnon H, Steinkellner S, Staehelin C, Gresshoff P, Vierheilig H (2007) Two defined alleles of the lrr receptor kinase *GmNARK* in supernodulating soybean govern differing autoregulation of mycorrhization. Physiol Plant 130:261–270

Morandi D (1996) Occurence of phytoalexins and phenolic compounds on endomycorrhizal interactions, and their potential role in biological control. Plant Soil 185:241–251

Nagahashi G, Douds DD (2005) Environmental factors that affect presymbiotic hyphal growth and branching of arbuscular mycorrhizal fungi. In: Declerck S, Strullu DG, Fortin JA (eds) *In vitro* culture of mycorrhizas. Springer, Heidelberg, pp 95–110

Norman J R, Hooker J E (2000) Sporulation of *Phytophthora fragariae* shows greater stimulation by exudates of non-mycorrhizal than by mycorrhizal strawberry roots. Mycol Res 104:1069–1073

Oka-Kira E, Kawaguchi M (2006) Long-distance signaling to control root nodule number. Curr Opin Plant Biol 9:496–502

Pearson JN, Abbott LK, Jasper DA (1993) Mediation of competition between two colonizing VA mycorrhizal fungi by the host plant. New Phytol 123:93–98

Pinior A, Wyss U, Piché Y, Vierheilig H (1999) Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Bot 77:891–897

Pozo MJ, Azcón-Aguilar C (2007) Unraveling mycorrhiza-induced resitance. Curr Opinion Plant Biol 4:393–398

Pozo MJ, Azcón-Aguilar C, Dumas-Gaudot E, Barea JM (1999) ß-1,3-glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. Plant Sci 141:149–157

Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM, Azcon-Aguilar C (2002) Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. J Exp Bot 53:525–534

Ryan A, Jones P (2004) The effect of mycorrhization of potato roots on the hatching chemicals active towards the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*. Nematol 6:335–342

Salzer P, Corbière H, Boller T (1999) Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus mosseae*. Planta 208:319–325

Scheffknecht S, Mammerler R, Steinkellner S, Vierheilig H (2006) Root exudates of mycorrhizal tomato plants exhibit a different effect on microconidia germination of *Fusarium oxysporum* f. sp. *lycopersici* than root exudates from non-mycorrhizal tomato plants. Mycorrhiza 16:365–370

Scheffknecht S, St-Arnaud M, Khaosaad T, Steinkellner S, Vierheilig H (2007) An altered root exudation pattern through mycorrhization affecting microconidia germination of the highly specialized tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is not tomato specific but also occurs in *Fol* non-host plants. Can J Bot 85:347–351

Singh R, Adholeya A, Mukerji KG (2000) Mycorrhiza in control of soil-borne pathogens. In: Mukerji KG, Chamola BP, Singh J (eds.) Mycorrhizal biology. Kluwer, New York pp 173–196

Smith S E, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Sood SG (2003) Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol Ecol 45:219–227

St-Arnaud M, Elsen A (2005) Interaction of arbuscular mycorrhizal fungi with soil-borne pathogens and non-pathogenic rhizosphere micro-organisms. In: Declerck S, Strullu DG, Fortin JA (eds) *In vitro* culture of mycorrhizas. Springer, Heidelberg, pp 217–231

St-Arnaud M, Vujanovic V (2007) Effect of the arbuscular mycorrhizal symbiosis on plant diseases and pests. In: Hamel C, Plenchette C (eds). Mycorrhizae in crop production: applying knowledge. Haworth, Binghampton, N.Y. (in press)

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1995) Altered growth of *Fusarium oxysporum* f. sp. *chrysanthemi* in an in vitro dual culture system with the vesicular arbuscular mycorrhizal fungus *Glomus intraradices* growing on *Daucus carota* transformed roots. Mycorrhiza 5:431–438

Trotta A, Varese GC, Gnavi E, Fusconi A, Sampo S, Berta G (1996) Interactions between the soil-borne root pathogen *Phytophthora nicotianae* var. *parasitica* and the arbuscular mycorrhizal fungus *Glomus mosseae* in tomato plants. Plant Soil 185–209

Vierheilig H (2004a) Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Bot. 82:1166–1176

Vierheilig H (2004b) Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. J Plant Physiol 161:339–341

Vierheilig H, Bago B (2005) Host and non-host impact on the physiology of the AM symbiosis. In: Declerck S, Strullu DG, Fortin JA (eds) *In vitro* culture of mycorrhizas. Springer, Heidelberg, pp 139–158

Vierheilig H, Ocampo JA (1990) Effect of isothiocyanates on germination of spores of *G. mosseae*. Soil Biol Biochem 22:1161–1162

Vierheilig H, Ocampo JA (1991) Receptivity of various wheat cultivars to infection by VA-mycorrhizal fungi as influenced by inoculum potential and the relation of VAM effectiveness to succinic dehydrogenase activity of the mycelium in the roots. Plant Soil 133:291–296

Vierheilig H, Piché Y (2002) Signalling in arbuscular mycorrhiza: facts and hypotheses. In: Buslig B, Manthey J (eds) Flavonoids in cell function. Kluwer, New York, pp 23–39

Vierheilig H, Alt M, Mohr U, Boller T, Wiemken A (1994) Ethylene biosynthesis and activities of chitinase and ß-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. J Plant Physiol 143:337–343

Vierheilig H, Garcia-Garrido MJ, Wyss U, Piché Y (2000a) Systemic suppression of mycorrhizal colonization in barley roots already colonized by AM-fungi. Soil Biol Biochem 32:589–595

Vierheilig H, Gagnon H, Strack D, Maier W (2000b) Accumulation of cyclohexenone derivatives in barley, wheat and maize roots in response to inoculation with different arbuscular mycorrhizal fungi. Mycorrhiza 9:291–293

Vierheilig H, Lerat S, Piché Y (2003) Systemic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonized by *Glomus mosseae*. Mycorrhiza 13:167–170

Villegas J, Williams RD, Nantais L, Archambault J, Fortin JA (1996) Effects of N source on pH and nutrient exchange of extramatrical mycelium in a mycorrhizal Ri T-DNA transformed root system. Mycorrhiza 6:247–251

Whipps JM (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bot 82:1198–1227

Wyss P, Boller, T, Wiemken A (1991) Phytoalexin response is elicited by a pathogen (*Rhizoctonia solani*) but not by a mycorrhizal fungus (*Glomus mosseae*) in bean roots. Experientia 47:395–399

Xavier LJC, Boyetchko SM (2004) Arbuscular mycorrhizal fungi in plant disease control. In: Arora DK (ed) Fungal biotechnology in agricultural, food, and environmental applications. Dekker, New York, pp 183–194

Resource Partitioning Between Extraradical and Intraradical AM Fungal Mycelium

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1 Introduction

The mycelia of arbuscular mycorrhizal (AM) fungi have an extraradical and an intraradical phase. Although these phases are connected and originate from the same individual, it is important to adopt a comparative approach when studying them. One should keep in mind that in nature a fungus usually consists of one extraradical mycelium (ERM) connecting several intraradical mycelia (IRM), which can be distributed between several plant species. Due to the simplicity of most experimental systems this aspect is often neglected. Furthermore, when a mycorrhizal fungus colonises a host plant it lives on the carbon (C) already present in the spore or, if it is a fungus that has already established its mycelium, in the mycelium. Upon colonization the new host provides a new C source and the newly fixed carbon is taken up and distributed in the mycelium. This fungal C allocation determines the mycelium growth and influences the C flow to the rhizosphere. Thus, even though the ERM and IRM originate from one organism, they cannot simply be considered as one entity.

The ERM is exposed to a highly variable soil environment, whereas the IRM develops in the root apoplast, which has a rather homogenous physico-chemical environment (Smith and Read 1997), although it will show some variation between plant species. It is possible that the IRM develops *Arum*-type colonization in one plant species and *Paris*-type in another. The factors controlling the formation of these morphological types are not known. The extraradical and intraradical phases differ clearly from each other in morphology and physiology. They differ in nutrient uptake and transfer, and are characterized by specific gene expression profiles (Balestrini and Lanfranco 2006). One way of evaluating the differences between the two phases is simply to compare the structures that form them: spores, runner hyphae, absorbing hyphae and in some cases auxiliary bodies of the ERM, with

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arbuscules, vesicles, coils, inter- and intraradical hyphae of the IRM. Another way to compare the two phases is to do quantitative biochemical measurements of roots (IRM) and soil (ERM) without considering particular structures.

In this chapter, we discuss the interplay between AM fungal ERM and IRM. We will do this by addressing the physiological aspects of nutrient partitioning. We will focus on the transport of C and phosphorus, as well as the enzymatic activity related to the fungal metabolism. We will link studies on AM fungal external phase with those on the internal phase, with emphasis on studies that include both ERM and IRM. We discuss the use of lipid and polyphosphate analyses as well as enzymatic activity staining as methods to study the difference between the two phases.

2 Mechanisms of Nutrient Transport

In the symbiotic stage of the AM fungus, plant-derived carbon is taken up by the IRM and translocated to the ERM, whereas mineral nutrients, especially phosphate, are taken up by the ERM, distributed over the ERM and translocated towards the IRM. This functional specialization implies that the AM fungus possesses a system for long-distance bidirectional transfer of large quantities of nutrients (Smith et al. 2001). The mechanisms that could be responsible for this transfer are summarized in Table 1 and discussed below.

Nutrient translocation through the ERM and IRM may take place via mass flow, which is a slow process, or via cytoplasmic streaming (Cooper and Tinker 1978, 1981). In the ERM of monoxenic root organ cultures, it is possible to see bidirectional cytoplasmic streaming, especially in runner hyphae. It is known that in ectomycorrhizal fungi translocation of nutrients occurs at high rates in this way (Finlay and Read 1986), and it might play an important role in AM fungi as well. It has also been shown that the cytoplasm in ERM of *Gigaspora margarita* contained acidic vesicles. These acidic vesicles moved bidirectionally as a component of cytoplasmic streaming, but their net rate of movement could not be determined (Saito et al. 2004).

Translocation of nutrients, such as phosphate (P), could also take place via transport in a motile pleiomorphic system of interlinked tubular vacuoles. These tubules form a reticulate system, and probably enable bidirectional translocation of different contents (Ashford and Allaway 2002). The main orientation of translocation is

Table 1 Mechanisms that can transfer nutrients within the AM fungal mycelium. A + indicates that the mechanism has been demonstrated and a ? that it is not yet demonstrated/determined. See text for details

Transfer mechanism	ERM	IRM	Direction
Cytoplasmic streaming	+	+	2-way
Acidic vesicles in cytoplasm	+	?	2-way
Tubular vacuoles	+	+	2-way
Lipid body movement	+	?	2-way, net direction towards ERM

longitudinal, suggesting its role in longitudinal translocation along hyphae (Cole et al. 1998). Such systems are observed in representatives of all major fungal groups including the ectomycorrhizal fungus *Pisolithus tinctorius* (Ashford et al. 1994; Rees et al. 1994; Allaway and Ashford 2001). It has been shown for this ectomycorrhizal fungus that the tubular vacuole system contained substantial amounts of phosphorus, which indicates a role in P translocation (Cole et al. 1998; Ashford et al. 1999; Ashford and Allaway 2002). The existence of a motile tubular vacuole system has also been shown to exist in germ tubes, in ERM and IRM of *Gigaspora margarita* (Uetake et al. 2002) and in ERM of *Glomus intraradices* (Olsson et al. 2002). The functioning of the tubular vacuole system of *Glomus intraradices* appeared to be inhibited by high P availability in the growth substrate (Olsson et al. 2002), indicating that the vacuolar system had a higher activity under conditions of nutrient limitation. The bidirectional cytoplasmic streaming of acidic vesicles and lipid bodies seemed to influence the tubular vacuole system of *Gigaspora margarita* (Saito et al. 2004).

An important part of the plant-derived carbon is transported towards the ERM, probably mainly in lipid bodies. This transport mechanism may prevent osmotic stress that otherwise could have been generated by the presence of large quantities of sugars (Smith et al. 2001). The movement of lipid bodies in the ERM of *Glomus intraradices* was highly variable but their maximum speed was not influenced by external temperature (Gavito et al. 2005). Still, carbon transfer to the fungus was reduced at low temperatures resulting in a reduced fungal growth. On the other hand, the phosphorus uptake and transfer to the plant was similar at 10° and 25°C (Gavito et al. 2005). The lipid bodies mainly moved with the cytoplasmic streaming, thus moving bidirectionally, and this resulted in a bidirectional translocation between IRM and ERM. However, it was suggested that their net movement is toward the growing tips of the ERM and that they are slowly consumed on this track (Bago et al. 2002b).

Thus, we expect that translocation of C and mineral nutrients (such as P) occurs in different systems. The major part of C is most likely translocated in lipid bodies and possibly by acid vesicles, whereas P is largely translocated in a system of tubular vacuoles. Another part of the transport of both nutrients can occur through cytoplasmic streaming and/or mass flow. These different mechanisms for translocation of nutrients are also indicators of hyphal vitality, and they respond to nutrient availability or external factors. They are important mechanisms for nutrient partitioning between the ERM and IRM in arbuscular mycorrhizal fungi.

3 Carbon Transport and Partitioning

3.1 Biomass Distribution

In today's ecosystem research, quantitative measurements get increasingly important due to the attention on the function of ecosystems in relation to the global nutrient cycles and the change in these cycles as a result of anthropogenic effects,

mainly in the context of global environmental change. The aim of this section is to show how quantitative estimates can be used to determine the total amount of AM fungal mycelia in soil, both the intraradical and the extraradical parts. We will make some tentative calculations on how large the extraradical mycelium can be compared to intraradical mycelia.

The methods normally used to measure IRM (proportion root length colonized) and ERM (hyphal length) do not allow comparison between the two phases. Instead biochemical signature measurements could be used (see Table 2). Chitin has been used in a few cases (Bethlenfalvay and Ames 1987) but has the disadvantage that it is present in other fungi, as well as in insects. Ergosterol is widely used as signature in ectomycorrhizal research, but it has been shown in several studies to be absent in AM fungi. Despite this fact, high performance liquid chromatography measurements of ergosterol were used as a means of estimating and comparing the fungal biomass of various AM fungi in soil and roots (Hart and Reader 2002). Their measurements probably mainly or only reflected saprotrophic fungi. A study by Olsson et al. (2003) has unambiguously shown that it should now be beyond question that AM fungi contain so little, or none at all of this compound, and that it is meaningless to use it as an indicator of AM fungal biomass. The best signature for the moment seems to be the neutral lipid fatty acid 16:1ω5. It is present in high amounts in AM fungi, with the exception of the genus *Gigaspora*. Furthermore, it is very specific to AM fungi. Ascomycetes and Basidiomycetes have a different fatty acid pattern dominated by 18-C fatty acids and fatty acid 16:0 (palmitic acid). The fatty acid 16:1ω5 is common in many bacterial groups, but neutral lipids are not an important storage compound in bacteria and the content of bacteria originated neutral lipid fatty acid 16:1ωw5 in soil is therefore very low. This also explains why phospholipid fatty acid 16:1ω5 is so insensitive as a signature for AM fungi, since the background originating from bacteria is very high in soil systems making this phospholipid fatty acid unusable as signature for ERM in soil systems/non-sterile systems, unless nonmycorrhizal controls have significantly lower contents. It can thus only be used in soil studies where it is possible to have nonmycorrhizal controls or in in vitro studies.

Table 2 Methods to quantify the biomass of intraradical and extraradical AM fungal mycelium. Their suitability (– = not suitable, + = suitable, ++ = very suitable) for the individual phases and their constraints are indicated

Biomass quantification method	ERM	IRM	Constraints
Hyphal length	+	–	Not suitable for IRM, for ERM spore biomass excluded
Root length colonization	–	++	Not suitable for ERM
Chitin	+	+	Present in other fungi and insects
Ergosterol	–	–	Not present in AM fungi
NLFA 16:1ω5	++	++	Low amount in *Gigaspora*
PLFA 16:1ω5	+	+	Insensitive in soils due to high background of bacteria

Even though there are good arguments that neutral lipid fatty acid 16:1ω5 is the best compound for comparing ERM to IRM, there is some important data from studies using other methods. Ronald Toth and Deborah Toth at the Northern Illinois University were the first to use morphometric quantification of root colonization. They squashed the root and studied each plant cell for fungal colonization (Toth and Toth 1982). Later, Toth et al. (1991) found that it was possible to relate common root colonisation estimation to fungal biomass, estimated with the chitin assay. Thereby a factor of 0.06 estimated the percent fungal biomass in roots from percent root colonisation (% colonization × 0.06 = % AM fungal biomass in roots). However, with the precaution that the estimation may not be relevant at very high colonisation percentages (such as over 80%). With their method a colonisation rate at 50% would mean that 3% of dry (mycorrhizal) root mass is fungus and 97% root tissue.

Neutral lipids are stored in lipid bodies in the mycelium and in spores, and it has been shown that these neutral lipids constitute a significant part of the fungal biomass and are thus relevant as biomass indicators. Bago et al. (2002b) used microscopical biovolume measurements and found that in hyphae of *Gigaspora rosea* between 2 and 36% was filled up by lipids. By using quantitative estimations of the fungal lipids, with nonmycorrhizal controls to subtract the background, Olsson et al. (1997) estimated that the proportion ERM of the total mycelium was between 50 and 75% in *Glomus caledonium*. The fungus was colonizing cucumber plants in pot culture, and it was found that the proportion of ERM decreased with P fertilization of the soil. This indicates that the mycelium partitioning by the fungus is regulated in similar way as the plants allocate their carbon resources. The shoot/root ratio decreases with less P availability, which means less allocation to the C absorbing part (the shoot) at low P. In a similar way, the fungus can be expected to allocate fewer resources to the C absorbing phase (the IRM) at low P availability (see the conceptual model in Fig. 1). The same relation can be viewed from an evolutionary or community perspective. By forming a large ERM, nutrients can be taken up efficiently and concentrated to the colonised roots. On the other hand, the formation of large IRM inhibits other fungi from colonizing the same root. Environments with lots of nutrients would select for fungi with large IRM, while nutrient poor, stress regulated, environments would select for fungi with large ERM. The proportion of ERM compared to IRM can be very high, but there are also indications that the root density in the soil decreases the proportion of ERM. In a study with *Glomus intraradices* and *Scutellospora calospora* colonizing *Plantago lanceolata,* the major part of both fungi was found in the roots (Van Aarle and Olsson 2003). The plants were grown in small pots where the soil was packed with roots. This may reflect that in natural grasslands, for example, where competition between plants is high, the major parts of the fungi are found as IRM (Fig. 1). By using the lipid signature for AM fungi this relation could be compared between different vegetation types.

It is clear that the biomass partitioning may differ between AM fungal species in particular at the species level. The *Scutellospora* and *Gigaspora* species do not form vesicles or intraradical spores, meaning that there is no major lipid storage

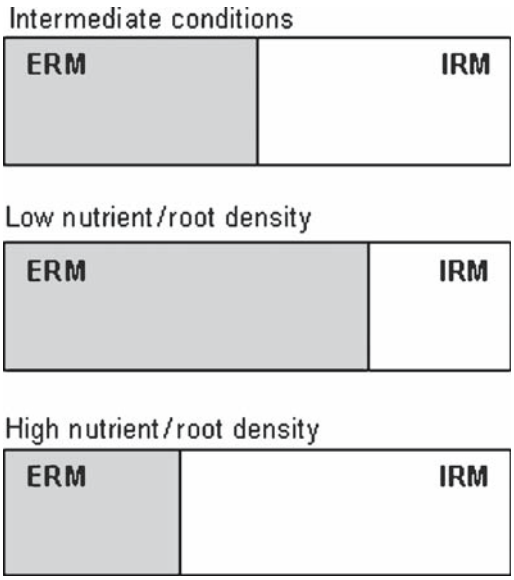

Fig. 1 A conceptual model for the biomass partitioning over the ERM (*grey*) and IRM (*white*). Under intermediate conditions, the biomass is approximately equally distributed (*top*). Under conditions of nutrient stress and/or low root density, i.e., in forest ecosystems, the larger part of the fungus is in the ERM (*middle*). However, under conditions of high nutrient availability and/or high root density, i.e., in most agricultural systems and natural grasslands, the larger part of the fungus is in the IRM (*bottom*)

within the colonized roots. When comparing *Glomus intraradices* with *Scutellospora calospora*, it was found that in the same system, the first fungus had higher proportion IRM than the second (Van Aarle and Olsson 2003). This fact could be due to the heavy formation of vesicles and intraradical spores in *Glomus intraradices*. In the same study, it was found that *Glomus intraradices* first formed vesicles, and then they were filled with lipids. This means that vesicle formation was not a good indication of storage levels.

3.2 Lipid Translocation

A prerequisite for effective partitioning of the ERM is a fast and precise lipid transport from the IRM. Just as plants may alter their shoot/root ratio, a two-phase organism such as an AM fungus can be expected to have evolved similar strategies for optimising the partitioning between the two phases. These strategies should include a lipid transport system since lipids are major carbon compounds, and since the ERM probably completely depends on the C transferred from the plants (Nakano et al. 1999). Olsson et al. (2002) calculated that 32% of labelled C in ERM of monoxenic

Glomus intraradices cultures was present in triacylglycerols. Also, glycogen is transported from IRM to ERM (Bago et al. 2003). The studies of lipids have, however, the advantage that the lipids have a specific fatty acid profile that differ AM fungal lipids from plant lipids. The most common fatty acids in Glominae as well as *Scutellospora* is 16:1ω5. This fatty acid can be analysed with high sensitivity using gas chromatography (Olsson 1999; Graham et al. 1995). *Gigaspora*, however, seems mainly to have a fatty acid profile more similar to plants – dominated by 18-C fatty acids (Graham et al. 1995) and it could therefore be difficult to apply the fatty acid technique in order to track these fungi. It is most probably so, that in most type of samples the signal to noise ratio for *Gigaspora* fatty acids is very low.

Indeed, the lipid movement in AM fungal hyphae is fast. By filming lipid body movement in living hyphae it is possible to estimate the speed of lipid movement. For *Glomus intraradices* in monoxenic cultures the speed was 4 μm/s and for *Gigaspora margarita* 8–11 μm/s (Gavito et al. 2005). This serves for a rapid translocation from IRM to ERM; within 24 h the lipids in *G. intraradices* can potentially have moved 35 cm (Bago et al. 2002b). Accordingly, the whole mycelium seems to act as one system for lipid distribution in which newly transferred plant assimilates are rapidly distributed throughout the mycelium. In pot systems where *P. lanceolata* was colonized by *Glomus intraradices* the carbon flow was studied using ^{13}C-labeled CO_2. By estimating the labeling in the specific AM fungal fatty acid 16:1ω5 in neutral lipids (including triacylglycerols) the timing of flow from IRM to ERM was estimated (Olsson and Johnson 2005). Large amounts of labeling was already detected in the ERM 2 days after labelling. At all time points sampled the labeling in IRM was almost exactly the same as in ERM. It does seem likely that lipid transport can support AM fungal mycelia at large distance from host roots. Only the hyphal tips seem to have a somewhat lower lipid content than the rest of the mycelium (Bago et al. 2002b).

When it comes to lipid content, the whole system of IRM and ERM seems to be like a system with free distribution of lipids within it. However, when it comes to uptake and synthesis the IRM is much more important. It is not only so that it is the IRM that is in contact with the C source, it is also so that hexoses can be taken up by the IRM, but not by the ERM (Pfeffer et al. 1999; Bago et al. 2002a). It may be that the enzymes needed for lipid synthesis are specifically expressed in the intraradical AMF structures (Trépanier et al. 2005). This fact makes the translocation of lipids even more important for the AM fungal growth.

4 Phosphate Transport and Partitioning

4.1 Phosphatase Enzymes

Phosphatases are important for most intracellular P transformations and they are also a part of the nutrition strategies. They can be exuded and thereby release phosphate from organic molecules, making it available for uptake. Plants usually exude

phosphatases, and it is believed that this strategy is an important part of P nutrition in plants. Plants do, however, only have phosphatases active at low pH values, and these acid phosphatases have probably little activity in more alkaline environments. AM fungi on the other hand contain both acid and alkaline phosphatases, just as do other fungi (Nahas et al. 1982; Bae and Barton 1989; Haas et al. 1992). High phosphatase activity has been detected both in intraradical (Gianinazzi et al. 1979; Van Aarle et al. 2002b) and extraradical (Van Aarle et al. 2001, 2002b; Olsson et al. 2002) AM fungal mycelium, but so far it seems that AM fungi do not excrete phosphatases very much (Joner et al. 2000) (Fig. 2). We will consider AM fungal phosphatases both in the aspect of intracellular P transport and transformations, and the fungal nutrition.

The intracellular phosphatase activity was examined in a number of studies, for example with the AM fungi *Glomus intraradices* and *Gigaspora margarita*. One interesting observation was that the alkaline phosphatase activity was higher than the acid phosphatase activity in the AM fungal ERM, while the opposite was true for the IRM (Van Aarle et al. 2002a, 2002b, 2005; Olsson et al. 2002). On the other hand, both alkaline and acid phosphatase activity were higher in the IRM than in the ERM of *Gigaspora margarita* (Van Aarle et al. 2002b) (Fig. 2).

We have already concluded that phosphatases in AM fungal ERM seem to be very little involved in exudation. A next question is then where they are located in the ERM. Joner and Johansen (2000) showed that, for the ERM of two *Glomus* isolates, a large part of the phosphatase activity was associated with the hyphal wall, whereas a smaller part was associated with internal structures. However, they did not work in sterile systems and it was not possible to separate AM fungal phosphatases from the phosphatase activity originating from bacteria associated with the hyphae. Enzymatic activity staining with the fluorescent ELF substrate indeed showed that some phosphatase activity was associated with the vacuoles (Olsson et al. 2002) whereas another part was associated with the hyphal wall (Van Aarle 2002). It has been hypothesized that plant cell wall-associated phosphatases may aid in the uptake

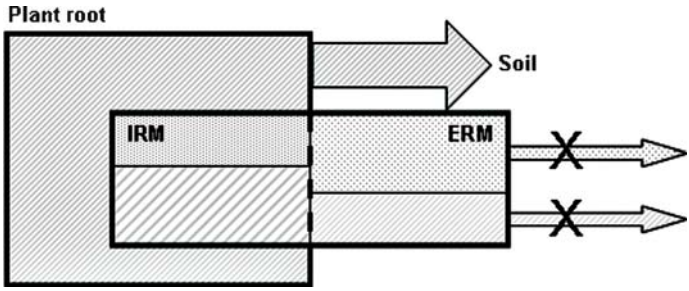

Fig. 2 A hypothetical model for the phosphatase activity of plant root, IRM and ERM. Acid phosphatase is indicated by the *hatched marking* and alkaline phosphatase by the *dotted marking*. A higher activity is indicated by a *heavier marking*. Active excretion of phosphatase is indicated by an *arrow* and passive release by a *crossed arrow*

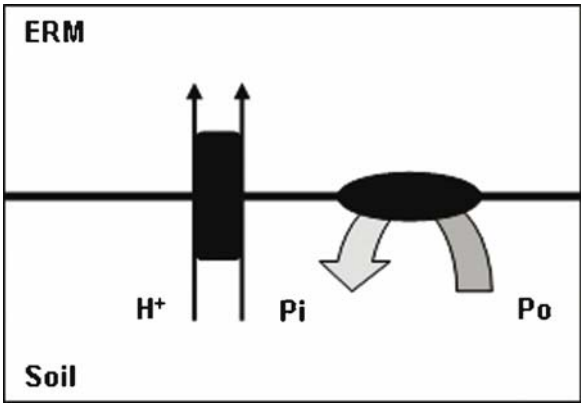

Fig. 3 Hypothetical role of hyphal wall-associated phosphatases. Phosphatase enzymes (*black oval*) located on the cell wall hydrolyse organic P (*Po*) compounds. Orthophosphate (*Pi*) can be directly transported into the ERM by proton co-transport (*black square*). *Thick line* indicates fungal membrane

of P from the soil or of P-esters that have leaked from the plant roots (Barrett-Lennard et al. 1993), and we believe that hyphal wall-associated phosphatases might have a similar recovery function. Because of their location, organic P compounds can be degraded close to the mycelium and orthophosphate could be taken up before physical immobilization or use by other microorganisms (Fig. 3).

The phosphatase activity in AM fungi is influenced by the external environment. Alkaline phosphatase activity associated with the ERM was, for example, inhibited by application of fungicides (Kjøller and Rosendahl 2000), increased pH (Van Aarle et al. 2002a) or simulated acid rain (Vosatka and Dodd 1998; Malcova et al. 1999). Furthermore, it was shown that the external nutrient availability may influence the phosphatase activity of the ERM (Olsson et al. 2002), and it could thus possibly also influence the P uptake of the AM fungus.

The location of phosphatases in the IRM indicates their involvement in the P transfer to the plant. The phosphatase activities have been localized in most structures (Gianinazzi et al. 1979; Ezawa et al. 1995; Van Aarle et al. 2002b, 2005) and these activities seem primarily located in the vacuoles (Gianinazzi et al. 1979; Gianinazzi-Pearson and Gianinazzi 1995; Saito 1995). However, the activities are highest in arbuscules and arbusculate coils (Van Aarle et al. 2005). These results support the role of these structures in the P transfer to the plant. Since alkaline phosphatase is mainly of microbial origin, it only appears in roots upon the development of an intraradical AM fungal mycelium. Alkaline phosphatase is considered as an important enzyme in metabolic processes that lead to P transfer to the host plant. Kojima and Saito (2004) related the phosphate efflux from IRM to fungal alkaline phosphatase activity, and their results suggested that fungal phosphatase may indeed be involved in the fungal P transfer to the plant. It was, furthermore, shown that alkaline phosphatase genes from *Glomus intraradices* and *Gigaspora*

margarita were constitutively expressed in mycorrhizal roots and their levels of transcripts were higher than those in ERM (Aono et al. 2004). Probably, alkaline phosphatase in the IRM has a role in the fungal P transfer to the plant, whereas in the ERM it may be involved in P uptake from the rhizosphere.

Alkaline phosphatase activity, visualized after staining with Fast blue, has previously been used as a marker for analyzing the symbiotic efficiency of AM fungal colonization (Tisserant et al. 1993). Acid phosphatase on the other hand has hardly been used for this, because the high background of acid phosphatase in root cells veils the activity of the IRM. Until recently, experimental constraints prevented an easy observation of the AM fungal acid phosphatase activity. However, it is possible to collect metabolically active IRM – succinate dehydrogenase activity could be shown after staining with NBT – after short enzymatic digestion of mycorrhizal roots (Saito 1995). This IRM can be studied for the proportion of acid phosphatase active structures (Van Aarle et al. 2002b). Furthermore, thin root sections in combination with ELF-staining techniques also allow the observation and quantification of acid phosphatase active structures (Van Aarle et al. 2005). Using this methodology, Van Aarle et al. (2005) could show that in both *Arum*- and *Paris*-type colonization of *Glomus intraradices* the acid phosphatase activity was more important than the alkaline phosphatase activity. In this study, it was shown that most of the mycorrhizal structures inside the roots showed succinate dehydrogenase activity, whereas about half of these IRM structures showed acid phosphatase activity and only a small part showed alkaline phosphatase activity. They furthermore observed that phosphatase activity was highest in arbuscules but that hyphal and arbusculate coils of the *Paris*-type colonization also showed an important phosphatase activity, whereas that the phosphatase activity in intercellular hyphae of the *Arum*-type colonization was low. For a summary of the different staining methods generally used to show general (succinate dehydrogenase) or specific (phosphatase) enzymatic activity of AM fungi, see Table 3. For detailed discussion of methods, see review of Vierheilig et al. (2005). In a study of extracted IRM of *Gigaspora margarita* from *Allium cepa* roots, the proportion of acid phosphatase active IRM and arbuscules were negatively correlated to the shoot P content under P-deprived conditions (Van Aarle et al. 2002b). No such correlations were found regarding the alkaline phosphatase active structures. Ezawa et al. (2001) found indications that acid phosphatase could be involved in the hydrolysis of vacuolar polyphosphate in intraradical hyphae upon which the inorganic P is released to the plant–fungal interface. The P efflux from extracted IRM was indeed equivalent to the amount of P produced by the hydrolysis of polyphosphate (Solaiman and Saito 2001). Thus, since the hydrolysis of polyphosphate is linked to the acid phosphatase activity, a high P transfer from fungus to plant is probably related to a high acid phosphatase activity in the IRM. This could mean that increased activity of acid phosphates in the IRM is related to an increased P transfer from fungus to plant, and that the location of acid phosphatase in IRM structures (such as arbuscules and coils) reflects the site of P transfer. In that case, acid phosphatase activity may be a suitable marker for AM fungal symbiotic efficiency, especially when its activity is higher than that of alkaline phosphatase.

Table 3 Overview of different staining methods frequently used to show metabolic activity or polyphosphate (poly-P) content of intraradical and extraradical AM fungal mycelium. The substrate is indicated first, followed by the enzyme for which it can be used. Their suitability (– = not suitable, + = suitable, ++ = very suitable) for the individual phases and their constraints are indicated

Staining method	ERM	IRM	Constraints
NBT – SDH	++	++	Counterstaining with acid fuchsin is preferable, especially for IRM
Fast blue – ALP	+	+	Counterstaining with acid fuchsin is preferable, especially for IRM
Fast blue – ACP	+	–	Sensitivity too low to show fungal specific ACP in the IRM
ELF – ALP	++	++	No counterstaining for fungal tissue found at present
ELF – ACP	++	++	High background staining of plant, thin root sections are preferable
Toluidine blue O – poly-P	++	++	Precipitation of poly-P with ethanol is preferable

NBT nitroblue tetrazolium, *SDH* succinate dehydrogenase, *ALP* alkaline phosphatase, *ACP* acid phosphatase, *ELF* enzyme-labeled fluorescence

4.2 Polyphosphate and Phosphate Partitioning

Inorganic phosphate that has been taken up by the AM fungal ERM is often accumulated before being translocated. Like in other fungi, accumulation of phosphate in the form of polyphosphate partly takes place in acidic, presumably vacuolar, compartments (Viereck et al. 2004). Polyphosphate is a linear polymer of phosphate residues that are linked by high energy bonds (Ogawa et al. 2000). Formation of polyphosphate maintains a low orthophosphate content in the mycelium, and it has a role in the promotion of long-term uptake and accumulation of phosphate (Ogawa et al. 2000). It is most likely that at least part of the fungal phosphate content is translocated in this form (Callow et al. 1978; Rasmussen et al. 2000). Viereck et al. (2004) characterized polyphosphate in the ERM of *Glomus intraradices* with ^{31}P NMR spectroscopy, and their data seemed to support the previous postulated hypothesis (Ashford and Allaway 2002; Ezawa et al. 2002; Van Aarle 2002) that polyphosphate is an important P metabolite which is translocated in a network of tubular vacuoles. Even though this polyphosphate is normally transferred towards the plant, it can be transferred bidirectionally, and it can be translocated within the ERM (Olsson et al. 2002) or to spores. Olsson et al. (2002) showed an accumulation of polyphosphate in ERM of *G. intraradices* which was obtained from medium without phosphate, when the fungus was grown in monoxenic root organ cultures. They visualized the polyphosphate content in the hyphae after precipitation with ethanol and subsequent staining with Toluidine blue O (see Table 3 for staining method).

The average chain length of polyphosphate in the ERM is 13–15 phosphate residues (Rasmussen et al. 2000; Viereck et al. 2004), but the polyphosphate chain is shorter in IRM (Solaiman et al. 1999; Viereck et al. 2004). This is consistent with

hydrolysis of polyphosphate in the IRM upon which the resulting orthophosphate is released into the apoplast of the plant-fungus interface (Ezawa et al. 2001). The net hydrolysis of polyphosphate in the ERM is however much weaker; there is more polyphosphate than there is degradation. There are strong indications that acid phosphatase is partly responsible for the hydrolysis of polyphosphate in the vacuoles of the IRM (see discussion in previous section). In the IRM of *Gigaspora margarita*, between 5 and 8% of the total P was present in the form of polyphosphate, whereas in the ERM between 8 and 17% was present in this form (Solaiman et al. 1999). This is equivalent to what was earlier reported for other AM fungi. *Gigaspora margarita* contained about 75% of its phosphate in the IRM, whereas about 25% was located in the ERM (Solaiman and Saito 2001). Figure 4 gives a schematic representation of the partitioning of phosphate and polyphosphate over the ERM and IRM of *Gi. margarita*. Over time, the amount of P distributed to the spores increased, but the total proportion of P contributed to the ERM did not change. These results support the hypothesis of a net P flux in the form of polyphosphate from the ERM towards the IRM. However, based on its relative low quantity it has to be kept in mind that its role might be limited.

Considerable amounts of P are taken up and transferred towards the plant, but AM fungi may differ in their strategy. Several indications are found that AM fungi from the Gigasporaceae can retain a large proportion of P in the mycelium and thus control the P transfer to the plant, whereas this is much less the case with fungi from the Glomeraceae. For *Scutellospora calospora* it has been shown that P accumulated in hyphae, but this was less for an *Acaulospora* and a *Glomus* isolate (Jakobsen et al. 1992b). The same authors observed in another study (1992a) that *Acaulospora* had the most extensive hyphal spread whereas that *Scutellospora* had most of its ERM close to the plant roots. Furthermore *Acaulospora* produced the largest increase in P uptake and plant growth (Jakobsen et al. 1992a). In the ERM of *Gigaspora rosea* an accumulation of polyphosphate was found, whereas this accumulation was not found for *Glomus manihotis* (Boddington and Dodd 1999). Furthermore, Solaiman and Saito (2001) observed a high P content in the mycelium of *Gigaspora margarita*, especially in the IRM. These observations indicate that either the P translocation or the P transfer to the host plant was delayed. Besides the amount of P that is translocated, the distance of translocation from the ERM to the host plant is dependent on the fungal isolate. Translocation distances of up to 7 cm for *Acaulospora* have been shown using ^{32}P tracers, whereas a *Glomus* species

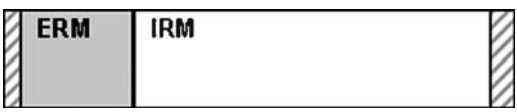

Fig. 4 The partitioning of P over the ERM (*grey*) and IRM (*white*) of *Gigaspora margarita*. The amount of P stored as polyphosphate is indicated by the *hatched areas*. Based on Solaiman et al. (1999) and Solaiman and Saito (2001)

could translocate only up to 4.5 cm and *Scutellospora* already had difficulties in reaching 2.5 cm (Jakobsen et al. 1992b). A high specific radioactivity in hyphae of *Scutellospora calospora* in comparison to that found in the hyphae of a *Glomus* and *Acaulospora* isolate indicates that the mycorrhizal fungal P transport from the soil to the plant is probably more limited by fungal translocation and transfer processes than by the fungal P uptake from the soil (Jakobsen et al. 1992b). This suggestion was confirmed in a study performed by Pearson and Jakobsen (1993). It thus seems very important to consider the fungal isolate or strategy before making general conclusions on AM fungal phosphate partitioning and transfer to the host plant.

5 Conclusions

AM fungi distribute their resources between the IRM and ERM. It seems that the translocation of triacylglycerols in lipid bodies and the translocation of P in tubular vacuoles are the most important mechanisms for the bidirectional transport in the symbiosis. The use of quantitative estimates of AM fungal fatty acids is a relevant way to estimate the biomass of AM fungi both in roots and in soil. Furthermore, by combining this technique to stable isotope labeling it is possible to estimate flow rates and retention of fungal C in IRM and ERM. A number of studies have shown that the fungal phosphatase activity is regulated in the fungal mycelium, and we conclude that this activity probably mainly reflects the internal P transitions in the mycelium, which is related to the overall P translocation and metabolism.

To further evaluate the regulation of AM fungal translocation capacity we suggest studies that specifically address the partitioning and differences between the IRM and ERM. Of particular interest is to compare different taxonomical groups of fungi. For example, do fungi with vesicles function in other ways than the fungi without? Perhaps even more interesting would be to investigate the partitioning in fungal mycelia that connects two, or more, host plants at the same time. Host plants that could be of different species, or exposed to different environmental treatments such as light or P.

References

Allaway WG, Ashford AE (2001) Motile tubular vacuoles in extramatrical mycelium and sheath hyphae of ectomycorrhizal systems. Protoplasma 215:218–225

Aono T, Maldonado-Mendoza IE, Dewbre GR, Harrison MJ, Saito M (2004) Expression of alkaline phosphatase genes in arbuscular mycorrhizal mycorrhizas. New Phytol 162:525–534

Ashford AE, Allaway WG (2002) The role of the motile tubular vacuole system in mycorrhizal fungi. Plant Soil 244:177–187

Ashford AE, Ryde S, Barrow KD (1994) Demonstration of short chain polyphosphate in *Pisolithus tinctorius* and the implication for phosphorus transport. New Phytol 126:239–247

Ashford AE, Vesk PA, Orlovich DA, Markovina A-L, Allaway WG (1999) Dispersed polyphosphate in fungal vacuoles in *Eucalyptus pilularis/Pisolithus tinctorius* ectomycorrhizas. Fungal Genet Biol 28:21–33

Bae KS, Barton LL (1989) Alkaline phosphatase and other hydrolyases produced by *Cenococcum graniforme*, an ectomycorrhizal fungus. Appl Environ Microbiol 55:2511–2516

Bago B, Pfeffer PE, Zipfel W, Lammers P, Shachar-Hill Y (2002a) Tracking metabolism and imaging transport in arbuscular mycorrhizal fungi. Plant Soil 244:189–197

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002b) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bago B, Pfeffer PE, Abubaker J, Jun J, Allen JW, Brouillette J, Douds DD, Lammers PJ, Shachar-Hill Y (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. Plant Physiol 131:1496–1507

Balestrini R, Lanfranco L (2006) Fungal and plant gene expression in arbuscular mycorrhizal symbiosis. Mycorrhiza 16:509–524

Barrett-Lennard EG, Dracup M, Greenway H (1993) Role of extracellular phosphatases in the phosphorus-nutrition of clover. J Exp Bot 44:1595–1600

Bethlenfalvay GJ, Ames RN (1987) Comparison of two methods for quantifying extraradical mycelium of vesicular-arbuscular mycorrhizal fungi. Soil Sci Soc Am J 51:834–837

Boddington CL, Dodd JC (1999) Evidence that differences in phosphate metabolism in mycorrhizas formed by species of *Glomus* and *Gigaspora* might be related to their life-cycle strategies. New Phytol 142:531–538

Callow JA, Capaccio LCM, Parish G, Tinker PA (1978) Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. New Phytol 80:125–134

Cole L, Orlovich DA, Ashford AE (1998) Structure, function and mobility of vacuoles in filamentous fungi. Fungal Genet Biol 24:86–100

Cooper KM, Tinker PB (1978) Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas II. Uptake and translocation of phosphorus, zinc and sulphur. New Phytol 81:43–52

Cooper KM, Tinker PB (1981) Translocation and transfer of nutrients in vesicular-arbuscular mycorrhizas IV. Effect of environmental variables on movement of phosphorus. New Phytol 88:327–339

Ezawa T, Saito M, Yoshida T (1995) Comparison of phosphatase localization in the intraradical hyphae of arbuscular mycorrhizal fungi, *Glomus* spp. and *Gigaspora* spp. Plant Soil 176:57–63

Ezawa T, Smith SE, Smith FA (2001) Enzyme activity involved in glucose phosphorylation in two arbuscular mycorrhizal fungi: indication that polyP is not the main phosphagen. Soil Biol Biochem 33:1279–1281

Ezawa T, Smith SE, Smith FA (2002) P metabolism and transport in AM fungi. Plant Soil 244:221–230

Finlay RD, Read DJ (1986) The structure and function of the vegetative mycelium of ectomycorrhizal plants II. The uptake and distribution of phosphorus by mycelial strands interconnecting host plants. New Phytol 103:157–166

Gavito ME, Olsson PA, Rouhier H, Medina-Penafiel A, Jakobsen I, Bago A, Azcon-Aguilar C (2005) Temperature constraints on the growth and functioning of root organ cultures with arbuscular mycorrhizal fungi. New Phytol 168:179–188

Gianinazzi S, Gianinazzi-Pearson V, Dexheimer J (1979) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localization of acid and alkaline phosphatase in onion roots infected by *Glomus mosseae* (Nicol. & Gerd.). New Phytol 82:127–132

Gianinazzi-Pearson V, Gianinazzi S (1995) Proteins and protein activities in endomycorrhizal symbioses. In: Varma A, Hock B (eds) Mycorrhiza, structure, function, molecular biology and biotechnology. Springer, Heidelberg, pp 251–266

Graham JH, Hodge NC, Morton JB (1995) Fatty acid methyl ester profiles for characterization of Glomalean fungi and their endomycorrhizae. Appl Environ Microbiol 61:58–64

Haas H, Redl B, Friedlin E, Stöffler G (1992) Isolation and analysis of the *Penicillium chrysogenum phoA* gene encoding a secreted phosphate-repressible acid phosphatase. Gene 113:129–133

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Jakobsen I, Abbott LK, Robson AD (1992a) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. I. Spread of hyphae. New Phytol 120:371–380

Jakobsen I, Abbott LK, Robson AD (1992b) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 2. Hyphal transport of ^{32}P over defined distances. New Phytol 120:509–516

Joner EJ, Johansen A (2000) Phosphatase activity of external hyphae of two arbuscular mycorrhizal fungi. Mycol Res 104:81–86

Joner EJ, Van Aarle IM, Vosatka M (2000) Phosphatase activity of extra-radical arbuscular mycorrhizal hyphae: a review. Plant Soil 226:199–210

Kjøller R, Rosendahl S (2000) Effects of fungicides on arbuscular mycorrhizal fungi: differential responses in alkaline phosphatase activity of external and internal hyphae. Biol Fertil Soil 31:361–365

Kojima T, Saito M (2004) Possible involvement of hyphal phosphatase in phosphate efflux from intraradical hyphae isolated from mycorrhizal roots colonized by *Gigaspora margarita*. Mycol Res 108:610–615

Malcová R, Vosatka M, Albrechtová J (1999) Influence of arbuscular mycorrhizal fungi and simulated acid rain on the growth and coexistence of the grasses *Calamagrostis villosa* and *Deschampsia flexuosa*. Plant Soil 207:45–57

Nahas E, Terenzi HF, Rossi A (1982) Effects of carbon source and pH on the production and secretion of acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) in *Neurospora crassa*. J Gen Microbiol 128:2017–2021

Nakano A, Takahashi K, Kimura M (1999) The carbon origin of arbuscular mycorrhizal fungi estimated from $\Delta^{13}C$ values of individual spores. Mycorrhiza 9:41–47

Ogawa N, DeRisi J, Brown PO (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in *Saccharomyces cerevisiae* revealed by genomic expression analysis. Mol Biol Cell 11:4309–4321

Olsson PA (1999) Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiol Ecol 29:303–310

Olsson PA, Johnson NC (2005) Tracking carbon from the atmosphere to the rhizosphere. Ecol Lett 8:1264–1270

Olsson PA, Bååth E, Jakobsen I (1997) Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Appl Environ Microbiol 63:3531–3538

Olsson PA, Van Aarle IM, Allaway WG, Ashford AE, Rouhier H (2002) Phosphorus effects on metabolic processes in monoxenic arbuscular mycorrhiza cultures. Plant Physiol 130:1162–1171

Olsson PA, Larsson L, Bago B, Wallander H, Van Aarle IM (2003) Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. New Phytol 159:7–10

Pearson JN, Jakobsen I (1993) Symbiotic exchange of carbon and phosphorus between cucumber and three arbuscular mycorrhizal fungi. New Phytol 124:481–488

Pfeffer PE, Douds Jr DD, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Rasmussen N, Lloyd DC, Ratcliffe RG, Hansen PE, Jakobsen I (2000) ^{31}P NMR for the study of P metabolism and translocation in arbuscular mycorrhizal fungi. Plant Soil 226:245–253

Rees B, Shepherd VA, Ashford AE (1994) Presence of a motile tubular vacuole system in different phyla of fungi. Mycol Res 98:985–992

Saito M (1995) Enzyme activities of the internal hyphae and germinated spores of an arbuscular mycorrhizal fungus, *Gigaspora margarita* Becker & Hall. New Phytol 129:425–431

Saito K, Kuga-Uetake Y, Saito M (2004) Acidic vesicles in living hyphae of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. Plant Soil 261:231–237

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Smith SE, Dickson S, Smith FA (2001) Nutrient transfer in arbuscular mycorrhizas: how are fungal and plant processes integrated? Aust J Plant Physiol 28:683–694

Solaiman MZ, Saito M (2001) Phosphate efflux from intraradical hyphae of *Gigaspora margarita in vitro* and its implication for phosphorus translocation. New Phytol 151:525–533

Solaiman MZ, Ezawa T, Kojima T, Saito M (1999) Polyphosphates in intraradical and extraradical hyphae of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. Appl Environ Microbiol 65:5604–5606

Tisserant B, Gianinazzi S, Gianinazzi-Pearson, Gollotte A (1993) *In planta* histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97:245–250

Toth R, Toth D (1982) Quantifying vesicular-arbuscular mycorrhizae using a morphometric technique. Mycologia 74:182–187

Toth R, Miller RM, Jarstfer AG, Alexander T, Bennet EL (1991) The calculation of intraradical fungal biomass from percent colonization of vesicular-arbuscular mycorrhizae. Mycologia 83:553–558

Trépanier M, Bécard G, Moutoglis P, Willemot C, Gagné S, Avis TJ, Rioux J-A (2005) Dependence of arbuscular-mycorrhizal fungi on their plant host for palmitic acid synthesis. Appl Environ Microbiol 71:5341–5347

Uetake Y, Kojima T, Ezawa T, Saito M (2002) Extensive tubular vacuole system in an arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 154:761–768

Van Aarle IM (2002) The ecophysiology of arbuscular mycorrhizal fungi: Phosphatase activity associated with extraradical and intraradical mycelium. PhD Thesis, Lund University, Lund

Van Aarle IM, Olsson PA (2003) Fungal lipid accumulation and development of mycelial structures in two arbuscular mycorrhizal fungi. Appl Environ Microbiol 69:6762–6767

Van Aarle IM, Olsson PA, Söderström B (2001) Microscopic detection of phosphatase activity of saprophytic and arbuscular mycorrhizal fungi using a fluoregenic substrate. Mycologia 93:17–24

Van Aarle IM, Olsson PA, Söderström B (2002a) Arbuscular mycorrhizal fungi respond to the substrate pH of their extraradical mycelium by altered growth and root colonization. New Phytol 155:173–182

Van Aarle IM, Rouhier H, Saito M (2002b) Phosphatase activities of arbuscular mycorrhizal intraradical and extraradical mycelium, and their relation to phosphorus availability. Mycol Res 106:1224–1229

Van Aarle IM, Cavagnaro TR, Smith SE, Smith FA, Dickson S (2005) Metabolic activity of *Glomus intraradices* in *Arum*- and *Paris*-type arbuscular mycorrhizal colonization. New Phytol 166:611–618

Viereck N, Hansen PE, Jakobsen I (2004) Phosphate pool dynamics in the arbuscular mycorrhizal fungus *Glomus intraradices* studied by *in vivo* ^{31}P NMR spectroscopy. New Phytol 162:783–794

Vierheilig H, Schweiger P, Brundrett M (2005) An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. Physiol Plant 125:393–404

Vosatka M, Dodd JC (1998) The role of different arbuscular mycorrhizal fungi in the growth of *Calamagrostis villosa* and *Deschampsia flexuosa*, in experiments with similated acid rain. Plant Soil 200:251–263

Ozone Stress and Ectomycorrhizal Root–Shoot Signaling

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1 Ozone Stress and Belowground Processes

Tropospheric ozone (O_3) is a secondary atmospheric pollutant, generated from oxides of nitrogen and volatile organic compounds reacting in the presence of sunlight. It has been recognized as an increasing and damaging agent to plants (Karnosky et al. 2005). The effects of O_3 on plant growth and development at different structural and functional levels of the aboveground parts have been studied extensively (Matyssek and Sandermann 2003), while the relative inaccessibility of plant roots and mycorrhiza has hampered efforts to understand effects of O_3 below ground (Andersen 2003; Schloter et al. 2005). O_3 triggers physiological changes in leaves that affect carbon source strength, i.e., the amount of carbon available for allocation to sink tissues. Decreased carbon assimilation, increased metabolic costs for repair mechanisms, and decreased phloem loading, all lead to decreased carbon allocation below ground, thus affecting roots, root symbionts, rhizodeposition, litter quality and quantity, and consequently the whole soil food web (Andersen 2003).

Ozone fumigation was shown to reduce total biomass of 3-year-old sugar maple seedlings, a species described as an intermediate to ozone-tolerant, grown in different light conditions (Topa et al. 2004), while a change in seedling biomass production in response to ozone fumigation was reported to occur between year 3 and 4 (Karnosky et al. 2005). Root plasticity has been proposed as the major mechanism by which plants cope with the naturally occurring heterogeneous supplies of nutrients in soil (Hodge 2004), and species-specific differences have been predicted using several specific root indices, such as specific root length (SRL, root length per unit mass). Thinner roots were reported to proliferate, i.e. show a fast response to a change in the environmental nutrient conditions (Hodge 2004). Carbon source-sink relationships or functional balance of roots and shoots were reported as primary factors in continuous adjustments between root and shoot growth (Tingey et al. 1996), possibly acting through root to shoot signaling, including hormonal

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regulation of root proliferation. In this context, the effects of ozone-fumigation on the cytokinins (CK) of beech trees (Winwood et al. 2007) can be related to mycorrhiza-associated changes in cytokinin concentrations in the host plants (Kraigher et al. 1991, 1993) and ozone-induced changes in fine root growth and ectomycorrhizal community structure (Grebenc and Kraigher 2007). The role of cytokinins in stress-related mycorrhiza-root-shoot signaling in ozone-fumigated beech trees and impacts of ozone fumigation on ectomycorrhizal root growth and community structure will be discussed.

2 Cytokinins and the Natural Environment

Plant hormones are naturally occurring substances effective at submicromolar levels that act as signals to stimulate or inhibit growth or regulate some developmental programme. Typically there is a specific regulated mechanism for the biosynthesis of the hormone as well as a regulated mechanism for its destruction. As a result, the levels of the hormone in the plant are modulated. Environmental factors can affect the biosynthesis, destruction and distribution of plant hormones, and in turn the hormones modify the developmental programme or the growth response. They act as agents by which information about the environment is distributed around the plant. The various plant parts are programmed to respond to a shift in phytohormone level by changes in gene expression that increase the chances that the individual will survive in the changing environment (Winwood et al. 2007).

Cytokinins, an intact adenine ring bearing an N^6-side chain, act as signaling hormones that play an essential role in regulating cytokinesis, growth and development in plants (Davies 2004). The variation and distribution of cytokinin species are not ubiquitous and depend on the plant species, tissue and developmental stage (Sakakibara 2004). Although the physiological function of individual compounds is not yet completely understood, they may be classified into three major, interconvertible groups: active forms – chiefly the purine free base; translocating forms – the ribosides and, in the phloem, ribotides; and storage; and stably inactivated forms – ribotides and conjugates such as glucosides. Isoprenoid cytokinins are biosynthesized by the isopentenylation of free adenine nucleotides, by the enzyme isopentenyltransferase (IPT). Plant IPT genes were first identified in *Arabidopsis*, whose genome encodes nine genes (*AtIPT1* to *AtIPT9*) which show similarity to the agrobacterial *ipt* gene. The environmentally-responsive accumulation of cytokinins has been reported in response to nitrogen availability, decapitation, long-daylight treatment, etc. *AtIPT3* expression can be correlated with the availability of inorganic macronutrients (nitrate, sulphate, phosphate ions; reviewed in Sakakibara 2004). Aromatic cytokinins have been identified in several plants, such as poplar (Strnad 1997), *Arabidopsis* (Tarkowská et al. 2003) and beech (Winwood et al. 2007). Among these, *meta*-topolin (mT) has the highest activity, and in addition to the free bases nucleosides, nucleotides and glucosides are also reported (Strnad 1997). Cytokinin degradation plays an important homeostatic role in the control of the accumulation of cytokinin metabolites and their distribution in plants. This

reaction is catalyzed by cytokinin oxidase/dehydrogenase (CKX), which irreversibly inactivates the cytokinin by cleaving the side chain.

The major sites of CK synthesis for export to the rest of the plant are root tips (Torrey 1976; Aloni et al. 2006). Many rhizospheric stresses (e.g., soil flooding or drying) perturb root cytokinin export, although their influence on growth may be masked by the export of other growth inhibitory signals, or altered nutrition (Dodd and Beveridge 2006). Numerous regulatory functions have been proposed for root-derived cytokinin, such as carrying nutritional information over long distances, and coordination of root and shoot development dependent on nitrogen availability (Schmülling 2002). Earlier investigations suggested that cytokinins could carry information about the nutritional status of organs (Wagner and Beck 1993; Schmülling 2002).

Nitrogen (N) supply also has root specific effects, namely regulation of root growth and architecture. High N concentration preferentially inhibits root growth and decreases the formation of lateral roots, while local application of nitrate to roots of nitrogen-limited plants leads to local proliferation of lateral roots (reviewed in Schmülling 2002), possibly through cytokinin-dependent regulation of root apical dominance (Aloni et al. 2006). From an ecological point of view, cytokinin-mediated root apical dominance, studied in *Arabidopsis*, gives priority to the primary root in competition with its own lateral roots as well as neighbouring root systems and enables the main root to reach water in deeper soil layers, which might be vital for plants before a dry period. Cytokinin regulates root architecture by balancing the promoting role of auxin in lateral root development. Cytokinin produced in the active tip of a primary root is the hormonal signal which enables maximum development of the growing primary root, retarding lateral root initiation. This reduces the quantity of lateral roots, the development of which would be at the expense of the primary root growth. By contrast, local stimulation by low NO_3^- and phosphate availability can induce lateral root initiation, promote lateral root elongation, an advantage in exploiting local variation in the availability of nutrients in the upper soil layers, and abolish cytokinin-dependent root apical dominance (Aloni et al. 2006).

From the sites of hormone production the signals move in specific structural pathways and by different mechanisms regulate plant growth and differentiation. The bulk of the CK synthesized in the root tips is exported through the xylem (Aloni et al. 2005). Leaf expansion and xylem cytokinin concentration (X-CK) decrease in response to N deprivation, although other studies indicate that direct leaf growth responses to N status of the soil can be independent of changes in X-CK supply to leaves (Rahayu et al. 2005). Therefore, X-CK probably acts in concert with a range of other, as yet unidentified signals (Dodd and Beveridge 2006). A major role of CKs has been shown to be long-distance signals mediating the shoot response to nitrate perception in roots (Rahayu et al. 2005), while neither abscisic acid (ABA) nor ethylene, the other two groups of phytohormones linked to leaf physiology, were found to be directly involved in the effects of N on leaf growth (Rahayu et al. 2005). In the roots, NO_3^- acts as a stimulator that increases the synthesis of two physiologically active forms of CK, zeatin (Z) and zeatin-riboside (ZR), and elevates the rate of the Z+ZR translocation in the xylem, which ultimately increases the rate of leaf growth.

In needles of Norway spruce trees of different damage classes, a positive correlation was found between the degree of damage and the concentrations of two cytokinin ribosides analyzed, namely ZR and isopentenyladenosine (iPAR) (von Schwartzenberg 1989; von Schwartzenberg and Hahn 1991). Elevated concentrations of these cytokinins were detected in the youngest needles with no visible damage. Unusually high levels of cytokinins were also found in needles of Norway spruce grown in polluted sites or on polluted substrates in Slovenia (Dent and Hanke 1995; Kraigher and Hanke 1996), as well as in Sitka spruce subjected to mist spraying with individual mineral components of aerial pollution, amongst which mineral N at neutral pH was the factor responsible (Collier et al. 2003a). Since roots are an important site for cytokinin biosynthesis in higher plants, the export of cytokinin ribosides from the root system in damaged trees might have increased. In this context, and in view of the changing mycoflora of polluted forest sites, the influence of ectomycorrhizal fungi on the content of cytokinins in plants must be taken into account.

3 Cytokinin Relations in Mycorrhiza

The first report of cytokinin production by mycorrhizal fungus was from Miller (1968). Cytokinins released into synthetic culture media by *Rhizopogon roseolus* were identified by physicochemical methods and bioassays as Z and ZR, while a third cytokinin occurred at the position of Zeatin ribotide (ZMP). Later, iPAR was also detected in the exudates of this fungus and its role as a precursor of ZR was suggested (Miura and Miller 1969). In 1971, Miller, using bioassays, reported on cytokinin production by *Amanita rubescens*, *Suillus cothurnatus, S. punctipes* and an unidentified ectendomycorrhizal species (Miller 1971). However, *Cenococcum graniforme (geophilum)* and *Thelephora terrestris* showed no cytokinin-like activity in the bioassays applied. Gogala (1971) also studied production of plant hormones by the ectomycorrhizal fungus *Boletus edulis* var. *pinicola* and found cytokinin-like substances in the medium. Two years later, Laloue and Hall (1973) and Miura and Hall (1973) confirmed that iPAR acts as a precursor of ZR and also reported on the presence of Ado-CO-thr (N-[9-(β-D-ribofuranosyl-9H) purin-6-ylcarbamoylthreonine), a tRNA component, in the fungal culture medium. In 1974, Crafts and Miller (1974) screened *Agaricus bisporus* and a series of *Rhizopogon* and *Hebeloma* species for cytokinin production. Of these, only *R. ochraceorubens* produced sufficient cytokinin to be detected by physicochemical methods and bioassays. An ectendotrophic mycorrhizal species and *Suillus punctipes* were tested and found to produce Z and ZR. In 1982, further screening was carried out by Ng et al. (1982) who reported on cytokinin production by *Rhizopogon luteolus, Boletus elegans* and *Suillus luteus*. Later, still using bioassays, combinations of hormones were investigated by Ho and Trappe (1987), who found a high level of variability among different isolates of the six *Rhizopogon* species they tested.

The effects of various culture conditions on cytokinins in cultures of some mycorrhizal fungi, all estimated by bioassays, were reported. Rudawska (1982) showed

negative effects of amino acids and urea on cytokinin production by *Suillus bovinus, S. luteus, Rhizopogon roseolus* and *Amanita muscaria*. However, Kampert and Strzelczyk (1989) later reported that different amino acids affected cytokinin production by *Rhizopogon luteolus* in different ways, although the effects were stimulatory in all cases. The cytokinins produced were Z and iPAR. *Suillus bovinus* was only found to produce cytokinins in the presence of alanine. These authors have also studied the effects of B-group vitamins (Strzelczyk and Kampert 1987) and of metabolites of actinomycetes (Strzelczyk et al. 1984) on cytokinin production by mycorrhizal fungi. Actinomycete metabolites stimulated production of iPAR and ZR by *Rhizopogon luteolus* and of iPAR, ZR and Z by *Paxillus involutus*. Pyridoxine stimulated cytokinin production by *R. luteolus* considerably, while *Suillus bovinus* only produced these substances in the presence of thiamine and biotin.

Twelve different ectomycorrhizal fungal species were tested for cytokinin production in liquid culture (Kraigher et al. 1991). All (*Amanita muscaria, Laccaria bicolour, L. laccata, L. proxima, Lactarius rufus, Hebeloma crustulliniforme, H. subsaponaceum, Paxillus involutus, Pisolithus tinctorius, Rhizopogon vinicolor, Suillus luteus, Thelephora terrestris*) secreted cytokinin into the medium (measured by RIA). Three (*L. bicolor, T. terrestris, S. luteus*) increased cytokinin production when grown in the presence of Norway spruce roots in the culture medium. Half-strength MS medium stimulated iPAR production (identified by HPLC-RIA and CI GC-MS) in *L. bicolor*, but not in *T. terrestris*. On the other hand, the strain of *T. terrestris* tested (strain no. 033 from D.J.Read's collection, obtained from Bush, UK, in 1986) was found to produce 10-times more cytokinins in pure cultures than any other fungi tested and was therefore referred to as a 'cytokinin overproducer' (Kraigher et al. 1991).

Cytokinins were found to stimulate mycelial growth of ectomycorrhizal fungi (Gogala 1971; Pohleven 1989; Sun and Fries 1992) and to stimulate ion transport and membrane permeability of ectomycorrhizal fungi (Pohleven 1989).

In 1980, Allen et al. (1980) reported increases in cytokinins in endomycorrhizal seedlings of *Bouteloua gracilis*, colonized by *Glomus fasciculatus*. Later, Dixon et al. (1988) observed that seedlings of *Citrus jambhiri*, colonized by *Glomus fasciculatum* or *G. mosseae* (but not *G. etunicatum*), showed significant increases in Z, ZR and dihydrozeatin in root exudates. In these studies, Z and ZR were analyzed by MS.

In ectomycorrhizal Douglas fir seedlings, xylem sap contents of ZR and ABA were analyzed (Coleman et al. 1990). For *Laccaria laccata, Hebeloma crustuliniforme* and *Thelephora terrestris*, none of the fungi influenced ABA concentrations, but all had an effect on ZR levels in the sap. The effects were higher with heavy *Hebeloma* and moderate *Thelephora* infection and lower in heavily *Laccaria*-colonized seedlings. The authors concluded that seedling metabolism could be influenced by the diversity in physiological effects of different fungal symbionts.

In needles of Loblolly pine (*Pinus taeda*), inoculated under sterile conditions with the mycorrhizal fungus *Pisolithus tinctorius*, 30–40% higher cytokinin contents were found than in nonmycorrhizal pine seedlings (Wullschleger and Reid 1990). In the same conditions, inoculation with the fungus *Suillus punctipes* had no detectable effect on cytokinin content or growth.

The ‘cytokinin overproducing’ strain of *Thelephora terrestris* (Kraigher et al. 1991; strain referred to as UK) and another strain of this fungus (isolated in a forest stand in North Slovenia, strain referred to as SI and producing approx. one-tenth of the cytokinin produced by the UK strain in pure cultures) affected the cytokinin content of needles of Norway spruce in dual cultures to different extents (Kraigher et al. 1993). The content of iPAMP, Z, ZR and iPAR was higher in needles of inoculated versus non-inoculated Norway spruce seedlings, while the iPA free base did not show any differences. Both strains raised the cytokinin content of the needles (iPAMP and Z) but with the UK strain the concentrations were up to 2.5-times higher than when inoculated with the SI-strain, a smaller difference than in pure culture exudates (Kraigher et al. 1993).

In considering the effects of mycorrhizal fungi on the cytokinin content of their plant symbionts, von Schwartzenberg and Hahn (1991) concluded that the elevated contents of cytokinins in spruce needles might reflect pollution-induced changes in species composition and abundance of ectomycorrhizal fungi in the root systems of these trees. However, there might be an additional influence of other soil microorganisms which have been shown to produce cytokinins, such as actinomycetes (Stevens and Berry 1988), rhizobia (Upadhyaya et al. 1991), bacteria (Kampert and Strzelczyk 1984) and others (review by Greene 1980). It was suspected that, since they are produced by such a range of soil microorganisms, cytokinins can be present in soil extracts in physiologically active amounts (van Staden and Dimalla 1976).

When Norway spruce seedlings were grown on sterilized or on nonsterile substrates (soils taken from two differently polluted forest stands in North Slovenia), statistically significant differences were found in the levels of iP-types of cytokinins (Kraigher and Hanke 1996). The presence of soil microorganisms appeared to influence the variability of the results (high variability in nonsterilized substrates, low in the sterilized ones) and the absolute concentrations of cytokinins detected in needles (higher concentrations in needles of seedlings, grown on nonsterile substrates than on those from the sterilized soil substrates). From the different concentrations of cytokinins in needles grown on substrates from the polluted and from unpolluted site, it was suspected that they might have been influenced by a shift in species and strain diversity of soil microorganisms, which was also detected in a parallel study of the diversity of types of ectomycorrhiza on Norway spruce in situ (Kraigher et al. 1996).

Arbuscular mycorrhizal (AM) plants have been reported to have enhanced CK accumulation in both shoots and roots, which is not a characteristic of pathogenic infections (Allen et al. 1980; Barea 1986; Drüge and Schonbeck 1992). A pathway defining the roles of CK in the establishment of the symbiotic phenotype, although not yet definitive, has been proposed (Barker and Tagu 2000). The question remains whether increased CK in mycorrhizal roots is of fungal or plant origin. In ectomycorrhizal plants, the existence of gradients of concentrations of signal molecules is known to be essential for gene regulation (Gay et al. 1994; Barker and Tagu 2000; Martin et al. 2001). In this both rhizospheric and plant (root) derived exudates trigger morphological and physiological changes in growth and development of mycorrhiza (Martin et al. 2001).

4 Ozone Effects on Roots and Ectomycorrhiza of Young and Adult Beech Trees

Exposure of trees to O_3 was found to modify the allocation of carbon to roots, which might disrupt root metabolism and rhizosphere organisms (Scagel and Andersen 1997). Despite the importance of forest soils in the global carbon cycle, fluxes of carbon associated with fundamental processes in forest soils and soil functional groups are inadequately quantified, limiting our understanding of carbon movement and sequestration in soils. Among the most important are mycorrhizal fungi which reduce overall retention of carbon in the plant–fungus symbiosis by increasing carbon in roots and belowground respiration and reducing its retention and release above ground (Rygiewicz and Andersen 1994). The mycelium of mycorrhizal fungi is the main linking network in the forest soil for the transport of water and nutrients, influencing the biodiversity and productivity of the whole ecosystem (Read 1998; Taylor and Alexander 2005). Mycorrhizae alter the size of belowground carbon pools, the quality and therefore the retention time of carbon below ground. In experimental conditions, it was shown that if elevated atmospheric CO_2 and altered climate stressors alter mycorrhizal colonization in forests, the role of forests in sequestering carbon could be altered (Rygiewicz and Andersen 1994).

Trees can form mycorrhiza with one or more types of ectomycorrhizae that can differ in morphological, physiological and ecological characteristics. Functional compatibility of mycorrhizal symbiosis depends on the species/strain of the fungus and the population of the tree species (Gianinazzi-Pearson 1984). Therefore, identification of the ectomycorrhizal community structure is of utmost importance for understanding of the functioning of the forest ecosystem and estimation of changes in them (Kraigher 1999; Kraigher et al. 2007). Different mycorrhizal fungi were found to release or to induce changes in cytokinin content in mycorrhizal seedlings (Kraigher et al. 1993; Kraigher and Hanke 1996), thus raising the possibility of changing root proliferation and development and subsequent carbon translocation to soils in forest ecosystem. In addition to ozone effects at the cellular and organ level, responses to the pollutant at the whole plant level, including plant-internal N- and C-cycling, are of special interest to estimate ozone toxicity in the long term. $\delta^{15}N$ is an important tool to provide insight into the N-cycle of plants and its connection to the carbon cycle. One of the most important factors influencing plant $\delta^{15}N$ is N-isotope fractionation during the transport of N-compounds from the soil to plant roots via ectomycorrhizal fungi (ECM). Isolated mycorrhizae are enriched in ^{15}N compared to different plant material of various species (Högberg 1999; Hobbie et al. 2005) and depleted in comparison to the source (Haberer et al. 2007).

Our aims were to assess possible changes in ectomycorrhizal communities in a novel experimental set-up located in the Kranzberg forest. The FACOS experiment (free-air canopy O_3 exposure system; http://www.casiroz.de) ensured chronic double ambient O_3 ($2\times O_3$) fumigation of adult beech trees in a 70-year-old mixed spruce–beech forest stand. We also analyzed roots in soil cores and assessed fine root

dynamics. Both parameters were analyzed for 2 years with respect to the increased O_3 concentration above ground and to the extreme drought summer conditions in the first year of sampling (2003; details presented in Grebenc and Kraigher 2007). In order to assess age-related differences in the reaction of beech plants to ozone fumigation, an additional experiment was established, using 4-year-old beech trees, planted in 30 L containers, which were exposed in the same conditions (for details see Železnik et al. 2007). It was hypothesized that ozone would: (1) reduce mycorrhization and ectomycorrhizal types diversity; (2) change ectomycorrhizal community structure in the differently exposed containers; (3) reduce root growth (biomass, number of root tips, root volume, specific root indices); and (4) change allocation of biomass between belowground and aboveground parts. We also predicted that light regime and ozone treatment interactions would be hard to establish since light regime was set at extremely low values, while ozone fumigation was limited to 2-fold (not exceeding 150 ppb) ambient ozone concentration.

4.1 Ozone Effects on Roots and Ectomycorrhiza of Adult Beech Trees

The effects of O_3 on belowground processes in a mature forest have only received limited attention so far. In the study, we have analyzed the community of ectomycorrhizal fungi and beech fine root dynamics over two growing seasons. In the experiment, two groups of five adult beech trees were either fumigated by 2× ambient ozone concentration or used as control. The main difference between previous studies and our approach was in that previous studies were performed on seedlings in pot experiments or in closed or open top chambers, and not in situ, in a mature forest stand. The ozone fumigation set-up was run for 3 years before the first samples were taken to avoid acute ozone effect to the belowground. Samples were then analyzed for the next 2 years.

Although beech is relatively irresponsive to tropospheric O_3, we found a pronounced effect of $2\times O_3$ on the number of vital ectomycorrhizal root tips and nonturgescent fine roots (named "Old ectomycorrhizae" in figures). Both categories of roots were significantly increased when compared to controls in two consecutive years at each sampling event (Fig. 1). The abundance of some types of ectomycorrhizae increased significantly (Fig. 2) while the species richness increased in 2004, but not in the extremely dry year 2003.

We hypothesized that the observed changes might be an expression of a transitional state in belowground succession of niches caused by O_3-induced effect on carbon allocation to roots and rhizosphere. We have detected changes in ectomycorrhizal species level, but Shannon-Weavers species diversity index and percentage of types of ectomycorrhizae did not change significantly in any sampling year, thus indicating our results cannot be unequivocally explained by summer drought in year 2003 or by O_3 exposure alone (Grebenc and Kraigher 2007).

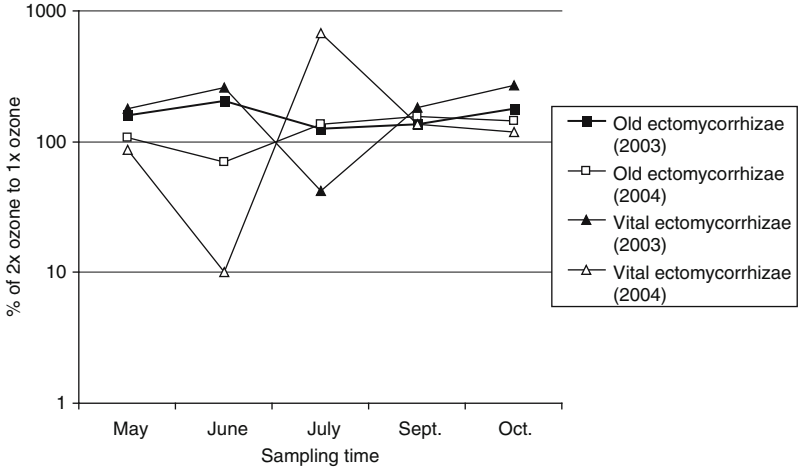

Fig. 1 Fine root turnover (vital ectomycorrhizae and old ectomycorrhizal roots) expressed as % $2\times O_3$ to $1\times O_3$. For most samplings the number of fine roots was higher in the $2\times O_3$ treatment

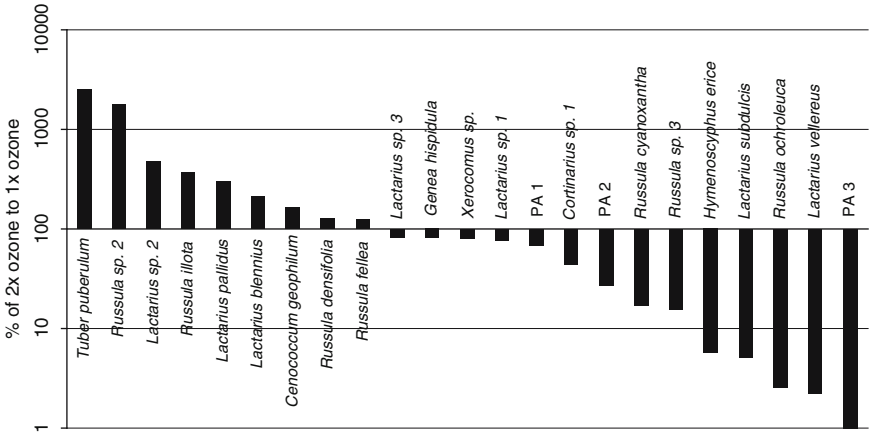

Fig. 2 Changes of ECM community structure from adult trees expressed in % of $2\times O_3$ to $1\times O_3$. Types of ectomycorrhizae only occurring under one of the analyzed treatments were excluded from this presentation

4.2 Ozone Effects on Roots and Ectomycorrhiza of Young Beech Trees

The effects of O_3 depend on the maturity-related physiological state of the plant; therefore, adult and young forest trees might react differently (Stroo et al. 1988; Bortier et al. 2000a, 2000b). To test the applicability of young beech plants for studying the effects of O_3 on forest trees and forest stands, beech seedlings were

planted in containers and exposed for 2 years to enhanced ozone concentration regime [ambient (control) and double ambient concentration, not exceeding 150 ppb] under different light conditions (sun and shade). After two growing seasons the biomass of the above- and belowground parts, beech roots (using WinRhizo® programme), anatomical and molecular identification of ectomycorrhizal types (ITS-RFLP and sequencing), and nutrient concentrations were assessed (for details, see Železnik et al. 2007). The mycorrhization of beech seedlings was very low (ca. 5% in shade, 10% in sun-grown plants), no trends were observed in mycorrhization (%) due to ozone treatment. The number of *Cenococcum geophilum*-type of ectomycorrhiza, as an indicator of stress in the forest stands, was not significantly different under different ozone treatments. *Cenococcum* occurred predominantly associated with sun-exposed plants, and was replaced as the major species by *Genea hispidula* under shade-grown plants. Different light regimes significantly influenced all parameters except shoot/root ratio and number of ectomycorrhizal types. In the ozone fumigated plants the number of types and their abundance (Fig. 3), number of root tips per length of 1–2 mm root diameter, root length density per volume of soil and concentration of Mg were significantly lower than in control plants (Železnik et al. 2007). Trends indicating a decrease were found in root, shoot, leaf and total dry weights, total number of root tips, number of vital mycorrhizal root tips, fine root (mass) density, root tip density per surface, root area index, concentration of Zn and Ca/Al ratio. Due to the general reduction in root growth indices and nutrient cycling in ozone fumigated plants, alterations in soil carbon pools could be predicted, leading to decreasing sequestration of carbon in soils.

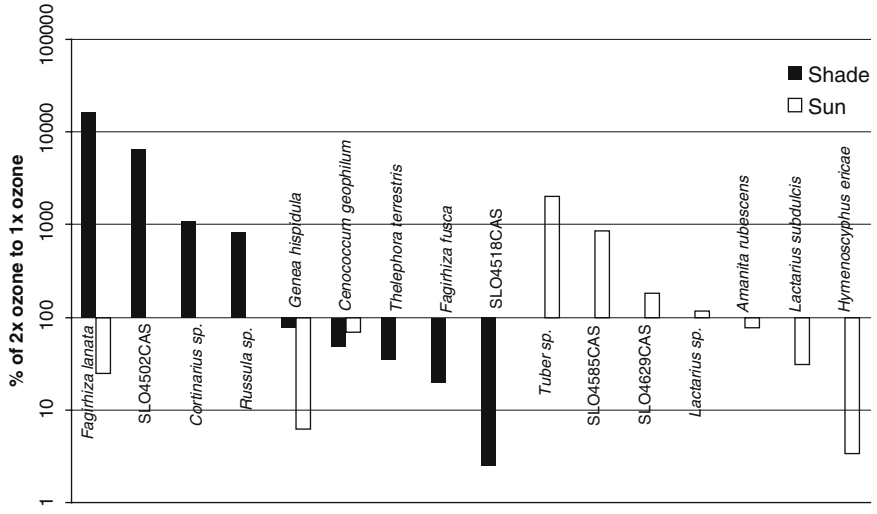

Fig. 3 ECM community structure (% $2\times O_3$ to $1\times O_3$)

4.3 Ozone Effects on CK Content of Leaves, Roots, Phloem and Xylem Sap of Adult Beech

Over the same period, the cytokinins of the mature trees were sampled and analyzed (Winwood et al. 2007). Beech proved to be unusual in that the naturally-occurring aromatic cytokinins, derivatives of benzyladenine and usually reported if at all as minor components, were as abundant as the isoprenoid types in leaves. Roots contained comparable levels but higher contents of the isoprenoid types than leaves. All groups of cytokinin, isopentenyladenine-types (iPA-CKs), zeatin-types (Z-CKs) and aromatic cytokinins (A-CKs), were affected in the same way by ozone treatment.

For shoots in the sun crown, leaf contents of cytokinin were lower for $2\times O_3$ trees than for $1\times O_3$ trees, and the effect was more marked later in the growing season. At the same time, the cytokinin content of phloem sap from sun crown shoots, almost entirely iPA-CKs, was substantially (2- to 3-fold) greater for $1\times O_3$ control trees than for the $2\times O_3$ treatment, i.e. there was a shortfall in phloem export as a results of elevated ozone, consistent with the observed lowering by ozone of the cytokinin content of sun crown leaves, the source of the phloem sap. For shoots down in the shade crown, no difference was detected. By contrast, root content and leaf xylem sap content of cytokinins were substantially (2- to 3-fold) higher for $2\times O_3$ trees than for $1\times O_3$ trees, and this effect was more marked earlier in the growing season.

In summary, mature beech trees chronically exposed to elevated ozone had more cytokinin in the roots, and the roots exported more to the leaves, especially early in the growing season. The expectation would be that the leaves in elevated ozone would therefore contain more cytokinin, yet they had less, especially later in the growing season, and were exporting less to the rest of the plant, including roots. The implication is that ozone-treated leaves are losing cytokinin faster, a loss that is partly compensated early in the season by increased import. Our results suggest that increased destruction of cytokinins in the presence of ozone is the mechanism for this loss because content, conjugation to glucosides, and export were all decreased, and import was increased, not decreased. The greater effect of ozone in the light is most likely due to increased stomatal aperture allowing ozone more access, but there may also be an involvement of light in ozone-mediated cytokinin destruction.

4.4 Ozone Effect to Fine Root Dynamics and Ectomycorrhizal Exploration Types in Scope of Cytokinin Level Changes

The experimental set-up installed in mixed beech–spruce forest in Kranzberg forest has allowed us to study the effects of chronically increased ozone concentration ($2\times O_3$) in canopies of adult (70 years old) trees, to belowground dynamics and symbiotic interactions (Grebenc and Kraigher 2007), and discuss the results obtained with respect to cytokinin distribution in the same adult beech trees (Winwood et al. 2007). The general effect of the increased ozone concentration to

fine root dynamics and ectomycorrhiza on a yearly scale was a permanent increase of the average number of vital and old ectomycorrhizal root tips in $2\times O_3$ treated trees compared to $1\times O_3$ treatment from June to October. The number increased with the decrease of cytokinin concentration, with the exception of $2\times O_3$ treatment in the extremely dry year (in 2003), when the number insignificantly decreased with the decrease of cytokinin content in xylem.

Comparing the change in abundance of particular ectomycorrhizal type relative to the cytokinin levels, we have established no significant correlation of the identified ectomycorrhizal types with changes in xylem or root cytokinin content. To overcome the problem of high number of species with low abundance, types of ectomycorrhizae were grouped into exploration types (Table 1). Different exploration types of ectomycorrhizae are distinguished based on the amount of emanating hyphae or presence and differentiation of rhizomorphs. They bear different ecological roles and ability to explore various areas of the soil and are thus assumed to exhibit different contributions to the trees (Agerer 2001).

In the Kranzberg forest plot five different exploration (sub)-types were identified with contact type, short-distance and medium-distance smooth sub-type as the most abundant (Fig. 4). For the purpose of presentation the data for all identified subtypes of medium-distance exploration types are combined.

The number of observed exploration types and their abundance did not differ significantly between the two sampling years (2003 and 2004; $p = 0.110$). From the statistical analysis (multifactor ANOVA) of abundance of exploration types correlated with ozone treatment in the plot, with year, and month of sampling as covariables, the ozone treatment showed significant increase of abundance of contact ($p = 0.0171$) and short-distance ($p = 0.0167$) exploration types. The medium–distance smooth subtype was significantly reduced ($p = 0.0002$) under $2\times O_3$ treatment in both years (Fig. 5).

The test of abundance of exploration types and cytokinin concentration in various parts of trees (analyzed with year of sampling, month of sampling and ozone treatment as co-variables) gave a less significant correlation (Table 2). Only the abundance of medium-distance smooth sub-type was significantly ($p = 0.0274$) influenced ($2\times O_3$ induced less iPA-CK and less of this exploration type, i.e., positive correlation among CK and exploration type) by isopentenyladenine-type cytokinins in phloem sap, and weakly significant ($p = 0.0488$) influenced (in $2\times O_3$ an increase of A-CK was found) by aromatic cytokinins content in roots (negative correlation with medium-distance smooth exploration type). In general the medium-distance smooth sub-type was influenced most by cytokinin content followed by the contact exploration type.

Exploration types in young beech seedlings showed the opposite reaction to increased ozone treatment from that of adult trees (Fig. 6a).

Here, the abundance of contact exploration types decreased with ozone treatment while the medium distance exploration types increased in abundance significantly. Changes in abundance of exploration types were not significant, and mainly limited to shade seedlings with only minor changes in sun-exposed seedlings. Absolute

Table 1 Grouping of types of ectomycorrhiza from Kranzberg into exploration types. Most types were ectomycorrhizal with adult beech trees, some with Norway spruce (marked with *PA1*, *2* or *3*)

Ectomycorrhizal exploration type	Types of ectomycorrhiza in Kranzberg forest stand occurring on adult and young beech plants, or on Norway spruce trees (PA – unidentified type on spruce)
Contact type	*Hymenoscyphus ericae*, *Lactarius subdulcis*, *Piceirhiza nigra*, *Russula fellea*, *Russula illota*, *Russula mairei*, *Russula xerampelina*, *Russula* sp. 1, *Russula* sp. 2, *Russula* sp. 3, *Russula* sp. 4, *Tomentella* sp 1, PA1, SLO4508CAS, SLO4518CAS, SLO4623CAS, SLO4526CAS, SLO4542CAS, SLO4552CAS, SLO4658CAS, SLO4565CAS, SLO4567CAS, SLO4570CAS, SLO4578CAS, SLO4588CAS, SLO4591CAS, SLO4618CAS, SLO4629CAS
Short-distance type	*Cenococcum geophilum, Fagirhiza setifera*, *Fagirhiza tubulosa*, *Genea hispidula*, *Phialophora* sp. 1, *Tuber puberulum*, *Tuber* sp. 1, PA2, PA3, SLO4530CAS, SLO4585CAS, SLO4610CAS, SLO4727CAS, SLO4799CAS
Medium-distance, fringe subtype	*Cortinarius* sp. 1, *Cortinarius* sp. 2, *Cortinarius* sp. 3, *Fagirhiza lanata*, *Fagirhiza fusca*, *Laccaria amethystina*, *Laccaria* sp. 1, SLO4537CAS, SLO4660CAS, SLO4754CAS
Medium-distance, smooth subtype	*Amanita rubescens, Lactarius acris*, *Lactarius blennius*, *Lactarius pallidus*, *Lactarius vellereus*, *Lactarius* sp. 1, *Lactarius* sp. 2, *Lactarius* sp. 3, *Russula ochroleuca*, *Thelephora terresttris*, SLO4502CAS, SLO4652CAS, SLO4723CAS
Long-distance type	*Paxillus involutus, Russula cyanoxantha*, *Russula densifolia*, *Xerocomus chrysenteron, Xerocomus* sp., SLO4515CAS

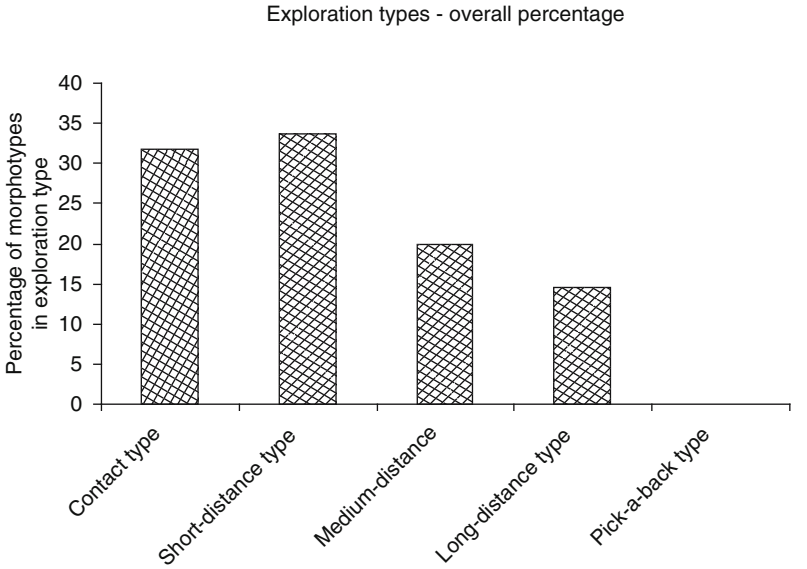

Fig. 4 Ectomycorrhizal exploration types of adult beech trees in Kranzberg research plot and their overall percentage in all samples

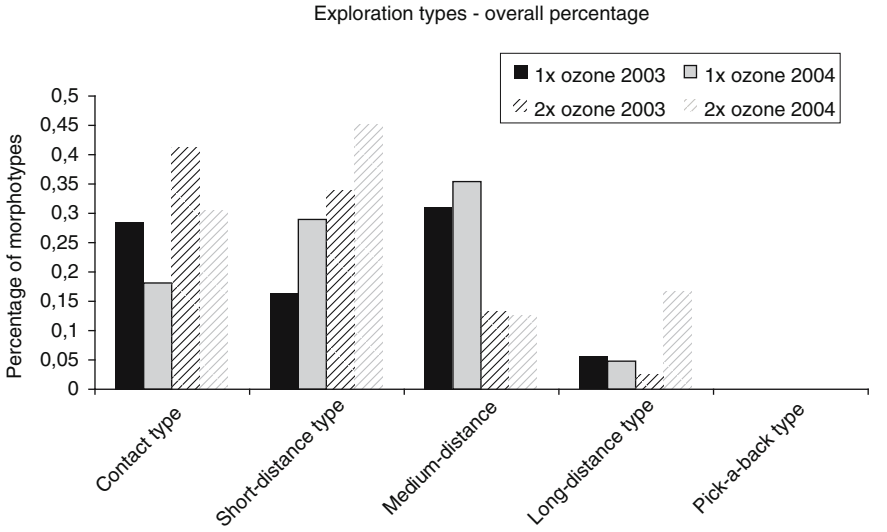

Fig. 5 The average share of exploration types in Kranzberg forest research plot with respect to the year of sampling and ozone treatment

Table 2 Form of CK and its location in plant organ with respect to the change in exploration type abundance. Only relations with MANOVA $p < 0.25$ were analyzed/shown

Form of CK location in plant organ	Exploration type	p value	CK concentration	Change in and its exploration type abundance
iPA-CK in leaf xylem sap	Contact type	0.2249	increasing	decreasing (except in very low and high CK conc.)
A-CK in roots	Contact type	0.2278	increasing	decreasing
iPA-CK in roots	Medium-distance, smooth subtype	0.1126	increasing	decreasing
A-CK in roots	**Medium-distance, smooth subtype**	**0.0488**	increasing	decreasing
iPA-CK in leaf xylem sap	Medium-distance, smooth subtype	0.1311	lower conc. higher conc.	increasing decreasing
Z-CK in leaf xylem sap	Medium-distance, smooth subtype	0.2061	lower conc. higher conc.	decreasing increasing
iPA-CK and Z-CK in leaf xylem sap	Medium-distance, smooth subtype	0.1613	increasing	increasing
iPA-CK in phloem sap	**Medium-distance, smooth subtype**	**0.0274**	increasing	increasing

numbers of ectomycorrhizal species per exploration type showed similar trends to their abundance except shade treated medium-distance exploration types (Fig. 6b).

Thus, the impact of ozone on young and adult beech trees was shown to be dependent on tree age-related physiology and its developmental phase related mycorrhizal community structure, which is in accordance with the differences reported in previous studies (Andersen 2003; Topa et al. 2004; Chung et al. 2006). Through the impacts on belowground species richness (Bell et al. 2005) and woody root dynamics (Pregitzer 2003) elevated ozone may differentially influence carbon losses from soils, which have been reported to be of the utmost importance in carbon sequestration (Schulze and Freibauer 2005; Bellamy et al. 2005).

5 Root–Shoot Signaling in Ozone Fumigated Beech Trees

Because ozone enters via stomata, leaves are the primary site of ozone interaction with the plant, effectively the 'site of perception' for responses to ozone, and the ozone-induced decrease in the cytokinin content of phloem sap in mature trees must be a downstream consequence of elevated ozone. Because roots are programmed to respond to cytokinins, phloem cytokinins arriving in the root influence root activity (Collier et al. 2003b). Cytokinins have a major influence on root/shoot ratio, promoting shoot development but strongly inhibiting root elongation and root branching (Riefler et al. 2006). The consequence of decreased phloem content of cytokinin

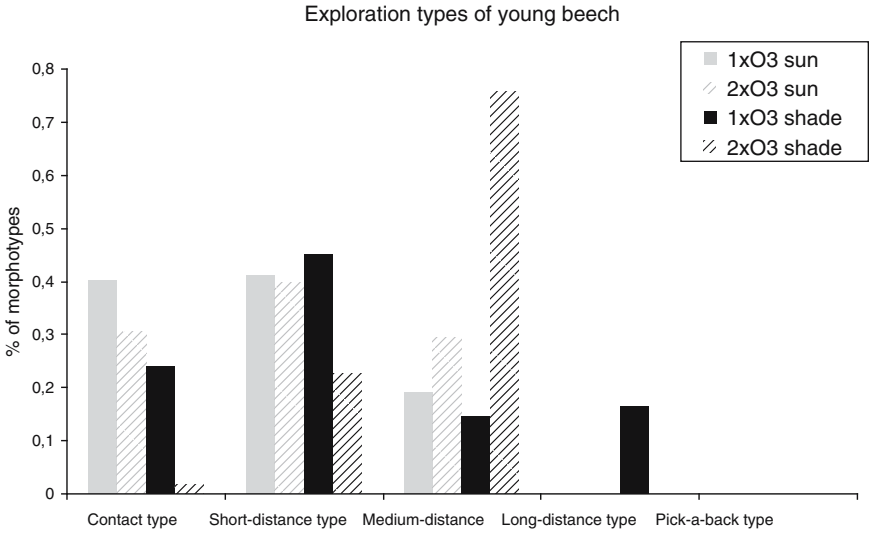

Fig. 6a Abundance of ectomycorrhizal exploration types of young beech trees in containers in Kranzberg research plot and their overall percentage in all samples

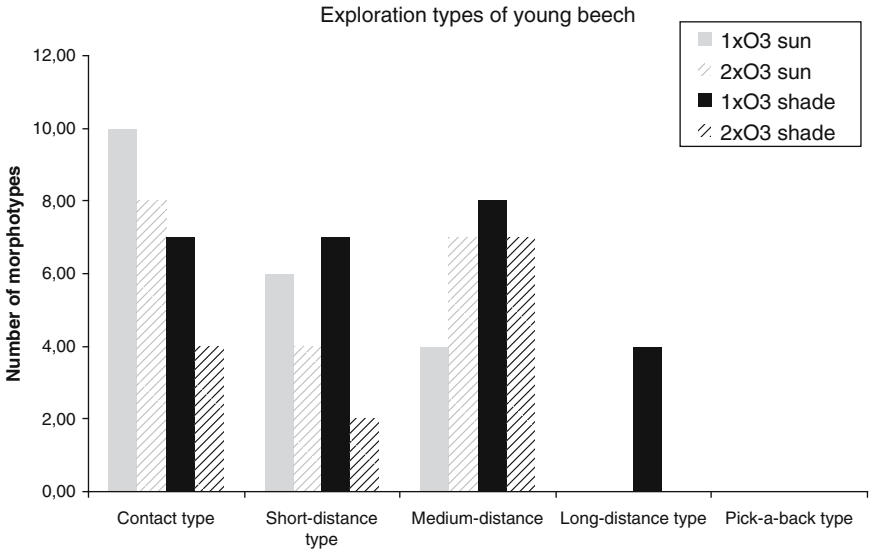

Fig. 6b Absolute number of ectomycorrhizal exploration types of young beech trees in containers in Kranzberg research plot and their overall percentage in all samples

is therefore predicted to be an increase in fine root development, which matches the observed effect. It may be that the lower levels of phloem cytokinins contribute to stimulating the formation of ECM. Cytokinins levels follow the mineral nutrient status of the plant closely, and acclimatory responses to mineral deficiency, of

which increased mycorrhization is one, are normally activated at low levels of hormone. The general trend of decreased phloem content of cytokinins coupled with increased abundance of ectomycorrhiza in response to elevated ozone is evidence consistent with this hypothesis. Paradoxically, the significant positive correlation between phloem sap cytokinin content and abundance of medium-distance exploration ECM type (see Section 4.1, Table 2) suggests that plant cytokinins arriving from the shoot in fact promote the formation of this ECM type. The correlation with the root content of aromatic cytokinins detected is of interest because these types make up a higher proportion of leaf CKs than root CKs, which suggests they may originate in the shoots and, like isopentenyladenosine, function as shoot-to-root signals to which fungal symbionts are programmed to respond.

Root tips are a major source of cytokinins, which are then distributed via the xylem (Aloni et al. 2005), and as discussed here there is good evidence that ectomycorrhizal fungi make a substantial contribution to the input of cytokinins at the roots. The ozone-induced increases in root tips and ECM are therefore predicted to result in increases in the cytokinin content of roots and xylem sap, which matches the observed effect. In spite of the additional cytokinin arriving in the leaf xylem of mature trees in elevated ozone, ozone-induced destruction in the leaves balances or even, for leaves in the sun crown, over-compensates the ozone-induced increase, leading to a correcting increase in root and ECM abundance (Fig. 7).

For other plants, including possibly the young beech trees, the balance of exchange between leaves and roots may not make such a positive contribution to

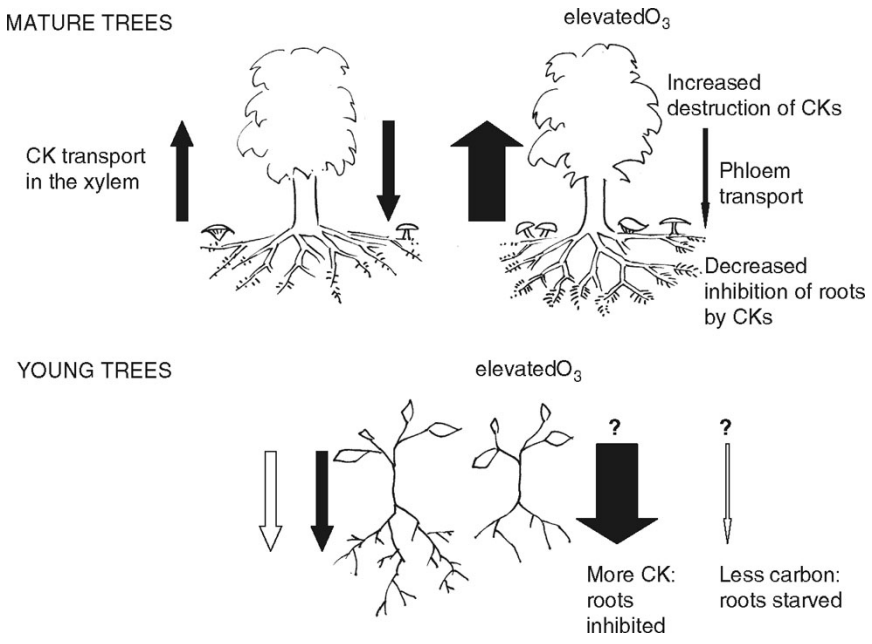

Fig. 7 Overall effects of mycorrhizal root to shoot to root signalling in ozone fumigated adult versus young beech trees/plants

survival. In types such as spruce for which leaves subject to pollution have elevated contents of cytokinin (von Schwartzenberg and Hahn 1991), the consequence will be increased cytokinin export via phloem, resulting in further suppression of root, and possibly ECM, development, as observed for young trees. Alternatively, ozone damage to the leaves of susceptible types may lower the supply of reduced carbon to the roots to such an extent that a positive response to lowered cytokinin is preempted (Fig. 7).

In the light of the opposite effects of ozone fumigation on mycorrhizal community structure and root growth indices in young beech plants (Železnik et al. 2007) and in adult trees (Grebenc and Kraigher 2007), the role of belowground processes provide insights of great ecological relevance. Forest ecosystems include complex mixtures of tree species, their developmental phases and belowground communities, in which mycorrhizal fungi are increasingly seen to have the potential to be the drivers of nutrient mobilization processes. The combination of stresses present in natural ecosystems, influencing plant hormonal signaling, such as drought (Pospišilová et al. 2000) in relation to depth-related fine root dynamics in dry periods (Mainiero and Kazda 2006), makes predicting O_3 effects difficult. Therefore, additional work is required for a better understanding of interactions between stresses, developmental phase-related physiology of trees, and their belowground complex-supporting components.

References

Agerer R (2001) Exploration types of ectomycorrhizae. A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. Mycorrhiza 11:107–114

Allen MF, Moore TSJr, Christensen M. (1980) Phytohormone changes in *Bouteloua gracilis* infected by vesicular mycorrhizae. I. Cytokinin increases in the host plant. Can J Bot 58:371–374

Aloni R, Langhans M, Aloni E, Dreieicher E, Ullrich CI (2005) Root synthesised cytokinin in *Arabidopsis* is distributed in the shoot by the transpiration stream. J Exp Bot 56:1535–1544

Aloni R, Langhans M, Aloni E, Ullrich CI (2006) Role of cytokinin and auxin in shaping root architecture: regulating vascular differentiation, lateral root initiation, root apical dominance and root gravitropism. Ann Bot 97:883–893

Andersen CP (2003) Source-sink balance and carbon allocation below ground in plants exposed to ozone (Tansley review). New Phytol 157:213–228

Barea JM (1986) Importance of hormones and root exudates in mycorrhizal phenomena. In: Gianinazzi-Pearson V, Gianinazzi S (Eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 177–197

Barker SJ, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbioses. J. Plant Growth Regul 19:144–154

Bell T, Newman JA, Silverman BW, Turner S, Lilley AK (2005) A contribution of species richness and composition to bacterial services. Nature 436:1157–1160

Bellamy PH, Loverland PJ, Bradley RI, Lark RM, Kirk GJD (2005) Carbon losses from all soils across England and Wales 1978–2003. Nature 437:245–248

Bortier K, Ceulemans R, de Temmerman L (2000a) Effects of tropospheric ozone on woody plants. In: Agrawal SB, Agrawal M (Eds) Environmental pollution and plant responses. CRC, Boca Raton, USA, pp 153–182

Bortier K, de Temmerman L, Ceulemans R (2000b) Effects of ozone exposure in open-top chambers on poplar (*Populus nigra*) and beech (*Fagus sylvatica*): a comparison. Environ Poll 109:509–516

Chung H, Zak DR, Lilleskov EA (2006) Fungal community composition and metabolism under elevated CO_2 and O_3. Oecologia 147:143–154

Coleman MD, Bledsoe CS, Smit B (1990) Root hydraulic conductivity and xylem sap levels of zeatin riboside and abscisic acid in ectomycorrhizal Douglas fir seedlings. New Phytol 115:275–284

Collier MD, Sheppard LJ, Crossley A, Hanke DE (2003a) Needle cytokinin content as a sensitive bioindicatior of N pollution in Sitka spruce. Plant Cell Environ 26:1929–1939

Collier MD, Fotelli MN, Nahm M, Kopriva S. Rennenberg H, Hanke DE, Gessler A (2003b) Regulation of nitrogen uptake by *Fagus sylvatica* on a whole plant level - interactions between cytokinin and soluble N compounds. Plant Cell Environ 26:1549–1560

Crafts CB, Miller C (1974) Detection and identification of cytokinins produced by mycorrhizal fungi. Plant Physiol 54:586–588

Davies PJ (2004) The plant hormones: their nature, occurrence, and functions. In: Davies PJ (Ed) Plant hormones. Biosynthesis, signal transduction, action! Kluwer, Dordrecht, pp 1–35

Dent RM, Hanke DE (1995) The effects of pollution on the cytokinin content of Norway spruce needles. In: Kraigher H, Batič F, Hanke DE, Agerer R, Grill D (Eds) Bioindication of forest site pollution: development of methodology and training. Slovenian Forestry Institute, Ljubljana, pp 89–93

Dixon RK, Garrett HE, Cox GS (1988) Cytokinins in the pressure exudate of *Citrus jambhiri* Lush. colonized by vesicular-arbuscular mycorrhizae. Tree Physiol 4:9–18

Dodd IC, Beveridge CA (2006) Xylem-borne cytokinins: still in search of a role? J Exp Bot 57:1–4

Drüge U, Schönbeck F (1992) Effect of Vesicular-arbuscular mycorrhizal infection on transpiration, photosynthesis and growth of flax (*Linum usitatissimum* L.) in relation to cytokinin levels. J Plant Physiol 141:40–48

Gay G, Normand L, Mermeisse R, Sotta B, Debaud JC (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. New Phytol 128:645–657

Gianinazzi-Pearson V (1984) Host-fungus specificity, recognition and compatibility in mycorrhizae. In: Verma DPS, Hohn Th (Eds) Genes involved in plant-microbe interactions. Springer, Heidelberg, pp 225–254

Gogala N (1971) Vloga rastnih substanc pri mikorizi med glivo *Boletus pinicola* Vitt. in borom *Pinus sylvestris* L. Razprave. Dissertationes XIV/5,. SAZU, Ljubljana

Grebenc T, Kraigher H (2007) Changes in community of ectomycorrhizal fungi and increased root turnover under adult beech trees chronically fumigated with double-ambient ozone concentration. Plant Biol 9:179–287

Greene EM (1980) Cytokinin production by microorganisms. Bot Rev 46:25–74

Haberer K, Grebenc T, Alexou M, Gessler A, Kraigher H, Rennenberg H (2007) Effects of long-term free-air ozone fumigation on $\delta^{15}N$ and total N in *Fagus sylvatica* and associated mycorrhizal fungi. Plant Biol 9:242–252

Ho I, Trappe JM (1987) Enzymes and growth substances of *Rhizopogon* species in relation to mycorrhizal hosts and infrageneric taxonomy. Mycologia 79:553–558

Hobbie EA, Jumpponen A, Trappe JM (2005) Foliar and fungal 15N:14N ratios reflect development of mycorrhizae and nitrogen supply during primary succession: testing analytical models. Oecologia 146:258–268

Hodge A (2004) The plastic plant: root response to heterogeneous supplies of nutrients (Tansley review). New Phytol 162:9–24

Högberg P (1999) 15N natural abundance in soil-plant systems. New Phytol 137:179–203

Kampert M, Strzelczyk E (1984) Effect of pH on production of cytokinin-like substances by bacteria isolated from soil, rhizosphere and mycorrhizasphere of pine (*Pinus silvestris* L.). Acta Microbiol Pol 33:77–85

Kampert M, Strzelczyk E (1989) Effect of amino acids on cytokinin-like substances production by mycorrhizal fungi of pine (*Pinus silvestris* L.). Agric Ecos Environ 28:219–228

Karnosky DF, Pregitzer KS, Zak DR, Kubiske M, Hendrey GR, Weinstein D, Nosal M, Percy KE (2005) Scaling ozone responses of forest trees to the ecosystem level in a changing climate. Plant Cell Environ 28:965–981

Kraigher H (1999) Diversity of types of ectomycorrhizae on Norway spruce in Slovenia. Phyton (Horn) 39(3):199–202.

Kraigher H, Hanke DE (1996) Cytokinins in Norway spruce seedlings and forest soil pollution. Phyton (Horn) 36:57–60.

Kraigher H, Grayling A, Wang TL, Hanke DE (1991) Cytokinin production by two ectomycorrhizal fungi in liquid culture. Phytochemistry 30:2249–2254

Kraigher H, Strnad M, Hanke DE, Batič F (1993) Cytokiningehalte von Fichtennadeln (*Picea abies* [L.] Karst) nach Inokulation mit zwei Stämmen des Mykorrhizapilzes *Thelephora terrestris* (Ehrh.) Fr Forstwiss Cbl 112:107–111

Kraigher H, Batič F, Agerer R (1996) Types of ectomycorrhizae and mycobioindication of forest site pollution. Phyton (Horn) 36:115–120

Kraigher H, Al Sayegh-Petkovšek S, Grebenc T, Simon i P (2007) Types of ectomycorrhiza as pollution stress indicators: case studies in Slovenia. Environ Monit Assess 128(1):31–45

Laloue M, Hall RH (1973) Cytokinins in *Rhizopogon roseolus*. Plant Physiol 51:559–562

Mainiero R, Kazda M (2006) Depth-related fine root dynamics of *Fagus sylvatica* during exceptional drought. For Ecol Manage 237:135–142

Martin F, Duplessis S, Ditengou F, Lagrange H, Voiblet C, Lapeyrie (2001) Developmental cross talking in the ectomycorrhizal symbiosis: signals and communication genes. New Phytol 151:145–154

Matyssek R, Sandermann H. (2003). Impact of ozone on trees: an ecophysiological perspective. Progr Bot 64:349–404

Miller CO (1968) Zeatin and zeatin riboside from a mycorrhizal fungus. Science 157:1055–1056

Miller CO (1971) Cytokinin production by mycorrhizal fungi. In: Proceedings of the First NACOM, Separate No. FS-294. USDA, pp 168–174

Miura G, Hall RH (1973) Trans-ribosylzeatin. Plant Physiol 51:563–569

Miura GA, Miller CO (1969) 6-(y,y-Dimethylallylamino) purine as a precursor of zeatin. Plant Physiol 44:372–376

Ng PP, Cole ALJ, Jameson PE, Mcwha JA (1982) Cytokinin production by ectomycorrhizal fungi. New Phytol 91:57–62

Pohleven F (1989) The influence of cytokinin 2iPA on growth, ion transport and membrane fluidity in mycelia of the mycorrhizal fungus *Suillus variegatus*. Agric Ecos Environ 28:399–402

Pospišilová J, Synková H, Rulcová J (2000) Cytokinins and water stress. Biol plant 43:321–328

Pregitzer KS (2003) Woody plants, carbon allocation and fine roots. New Phytol 158:421–424

Rahayu YS, Walch-Liu P, Neumann G, Römheld V, von Wirén, N, Bengerth F (2005) Root-derived cytokinins as long-distance signals for $NO^{-}3$-induced stimulation of leaf growth. J Exp Bot 56:1143–1152

Read DJ (1998) Plants on the web. Nature 396:22–23

Riefler M, Novak O, Strnad M, Schmülling T (2006) *Arabidopsis* cytokinin mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell 18:40–54

Rudawska M (1982) Effect of various organic sources of nitrogen on the growth of mycelium and content of auxin and cytokinin in cultures of some mycorrhizal fungi. Acta Physiol Plant 4:11–20

Rygiewicz PT, Andersen CP (1994) Mycorrhizae alter quality and quantity of carbon allocated below-ground. Nature 369:58–60

Sakakibara H (2004) Cytokinin biosynthesis and metabolism. In: Davies PJ (ed) Plant hormones. Biosynthesis, signal transduction, action! Kluwer, Dordrecht, pp 95–114

Scagel CF, Andersen CP (1997) Seasonal changes in root and soil respiration of ozone-exposed ponderosa pine (*Pinus ponderosa*) grown in different substrates. New Phytol 136:627–643

Schloter M, Winkler Aneja JBM, Koch N, Fleischmann Pritsch K, Heller W, Stich S, Grams TEE, Göttlein A, Matyssek R, Munch JC (2005) Short term effect of ozone on the plant-rhizosphere-bulk soil system of young beech trees. Plant Biol 7:728–736

Schmülling T (2002) New insights into the functions of cytokinins in plant development. J Plant Growth Regul 21:40–49

Schulze D-E, Freibauer A (2005) Carbon unlocked from soils. Nature 437:205–206

Stevens GAJr, Berry AM (1988) Cytokinin secretion by *Frankia* sp. HFPArI3 in defined medium. Plant Physol 87:15–16

Strnad M (1997) The aromatic cytokinins. Physiol Plant 101:674–688

Stroo HF, Reich PB, Schoettle AW, Amundson RG (1988) Effects of ozone and acid rain on white pine (*Pinus strobus*) seedlings grown in five soils. II. Mycorrhizal infection. Can J Bot 66:1510–1516

Strzelczyk E, Kampert M, Michalski L (1984) Production of cytokinin-like substances by mycorrhizal fungi of pine (*Pinus sylvestis* L.) in cultures with and without metabolites of actinomycetes. Acta Microbiol Pol 34:177–186

Strzelczyk E, Kampert M (1987) Effect of B-group vitamins on cytokinin-like substances production by ectomycorrhizal-fungi of pine (*Pinus sylvestris* L.). Symbiosis 3: 135–146

Sun Y-P, Fries N (1992) The effect of tree-root exudates on the growth rate of ectomycorrhizal and saprotrophic fungi. Mycorrhiza 1:63–69

Tarkowská D, Doležal K, Tarkowski P, Åstot C, Holub J, Fuksová K, Schmülling T, Sandberg G, Strnad M (2003) Identification of new aromatic cytokinins in *Arabidopsis thaliana* and *Populus* x *Canadensis* leaves by LC-(+)ESI-MS and capillary liquid chromatography/frit-fast atom bombardment mass spectrometry. Physiol Plant 117:579–590

Taylor AFS, Alexander I (2005) The ectomycorrhizal symbiosis: life in the real world. Mycologist 19:102–112

Tingey DT, Johnson MG, Phillips DL, Johnson DW, Ball TJ (1996) Effects of elevated CO_2 and nitrogen on the synchrony of shot and root growth in ponderosa pine. Tree Physiol 16:905–914

Topa MA, McDermitt DJ, Yun S-C, King PS (2004) Do elevated ozone and variable light alter carbon transport to roots in sugar maple? New Phytol 162:173–186

Torrey JG (1976) Root hormones and plant growth. Annu Rev Plant Physiol 27:435–459

Upadhyaya NM, Letham DS, Parker CW, Hocart CH, Dart PJ (1991) Do rhizobia produce cytokinins? Biochem Int 24:123–130

Van Staden J, Dimalla G (1976) Cytokinins from soils. Planta 130:85–87

Von Schwartzenberg K (1989) Der cytokiningehalt in nadeln unterschiedlich stark von 'Neuartigen Waldschäden' betroffener fichten (*Picea abies* (L.) Karst.), bestimmt mittels einer immunoenzymatischen methode - ELISA. Inaugural Dissertation, Rheimischen-Friedrich-Wilhelms Universität Bonn

Von Schwartzenberg K, Hahn H (1991) The cytokinin content in needles of Norway spruce (*Picea abies* [L.]. Karst) with different degrees of damage. J Plant Physiol 139:218–223

Wagner BM, Beck E (1993) Cytokinins in the perennial herb *Urtica dioica* L. as influenced by its nitrogen status. Planta 190:511–518

Winwood J, Pate AE, Price AJ, Hanke EE (2007) Effects of long-term, free-air ozone fumigation on the cytokinin content of mature beech trees. Plant Biol 9:265–278

Wullschleger SD, Reid CPP (1990) Implication of ectomycorrhizal fungi in the cytokinin relations of loblolly pine (*Pinus taeda* L.). New Phytol 116:681–688

Železnik P, Hrenko M, Then Ch, Koch N, Grebenc T, Levani T, Kraigher H (2007) CASIROZ: root parameters and types of ectomycorrhiza of young beech plants exposed to different ozone and light regimes. Plant Biol 9:298–308

From Production to Application of Arbuscular Mycorrhizal Fungi in Agricultural Systems: Requirements and Needs

Jacqueline Baar

1 Introduction

Growing crops in sustainable systems with reduced chemical inputs has increasing interest. In low-input systems, the main requirements for crops to achieve high and profitable yields are sufficient uptake of nutrients and coping with unpredictable and varying levels of soil nutrients. The application of beneficial microorganisms can enhance the growth of crops and increase their yields. An important group of these beneficial microorganisms include the symbiotic living arbuscular mycorrhizal (AM) fungi that play a vital role in the acquisition of mineral nutrients from soils, stimulating plant development. Also, these beneficial symbiotic fungi can suppress the development of below- and aboveground plant pathogens, resulting in better plant performance.

In the last few decades, interest in AM fungi has increased. The production and application of these beneficial fungi for agricultural purposes has grown over the last decades worldwide; companies are producing mycorrhizal fungi and their number is rising. The application of AM fungi has enormous potential for large-scale agricultural systems and can be beneficial in sustainable production of main crops contributing to reduced input of chemical fertilizers and pesticides.

For successful application of AM fungi with economically profitable results, the environment must be suitable for the development of AM fungal symbiosis. In this chapter, the main environmental factors affecting the development of mycorrhizal symbiosis are discussed. Moreover, various approaches enabling possibilities to increase the application of mycorrhizal fungi are addressed.

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2 Basic Requirements for Sustainable Agricultural Systems

The majority of crops in agricultural systems are currently grown with significant input of mineral fertilizers and chemical pesticides. This results in considerable damaging losses to the environment and is in contrast with sustainability. Growing crops in more low-input systems with reduced chemical inputs is gaining ground. Sustainable cropping can be achieved by reducing inputs of chemicals and the replacement of mineral fertilizers and chemical pesticides by biological alternatives leading to the production of healthier food which benefits the consumers and reduces health risks (Van Bruggen 1995; Atkinson et al. 2002).

The recognition of a need to foster sustainable agriculture systems with reduced inputs of chemical fertilizers and pesticides is not new, and has been made more imperative because of the recognition that highly specialized, capital-intensive and chemical-intensive agricultural methods, that boosted production outputs to higher and higher levels, have a myriad of adverse effects on natural resources, environmental quality, human health and food quality and safety. Therefore, increasing numbers of producers (i.e., farmers) in Europe have tended to move towards sustainable low-input agricultural practices, pushed by the cost/price squeeze of food production and the increased dependence on off-farm chemical inputs.

Plant strategies for sustainable systems, such as low-input organic agricultural systems, have been outlined by Lammerts Van Bueren. The main requirements for crops to achieve high and profitable yields in such sustainable systems are to scavenge for nutrients and to cope with unpredictable and varying levels of soil nutrients (Lammerts Van Bueren 2002). The application of beneficial microorganisms can enhance the growth of crops and increase their yields.

For a plant cultivar oriented to sustainable agriculture approach, the main requirements are adaptation to soil fertility properties and reduced input of fertilization. Recently, scientists from all over the world have started to understand that microbial biodiversity is a crucial factor regulating soil processes in sustainable agricultural systems, including low-input systems, and specifically in that portion of soil influenced by roots, i.e., the rhizosphere (Copley 2000). Within the rhizosphere, beneficial micro-organisms are key active components of the overall biodiversity, assisting plant growth through their involvement in the processes of nutrient cycling and acquisition, antagonism of detrimental organisms, and enhancement of soil structure (Visser and Parkinson 1992; Schimel 1995). An important group of these beneficial microorganisms include arbuscular mycorrhizal (AM) fungi. These beneficial symbiotic fungi play a vital role in the acquisition of mineral nutrients from soils, and facilitating plant development, particularly in agricultural systems with low inputs of chemical fertilizers and fungicides (Estaún et al. 2002; Ryan and Graham 2002). Also, AM fungi can suppress the development of below- and aboveground plant pathogens, resulting in better crop performance (Smith and Read 1997).

In the last few decades, many scientists in Europe have been working to increase knowledge about the role of AM fungi in agricultural systems. The reason for the interest in these mutualistic living symbionts is harnessing the beneficial

functions of these organisms in agricultural systems shows enormous potential to reduce chemical inputs is contributing to increased sustainability. Also, the interest in production of AM fungi for agricultural purposes has grown over the last decade (Vosatka and Dodd 2002). Several companies in Europe have started with commercial production of these fungi for application in agricultural systems. This has resulted in the availability of AM fungal inoculum on a large scale.

AM fungi have enormous potential enhancing productivity and quality of crops in combination with reduction of applications of mineral phosphate fertilizers; this could be of considerable economic advantage for producers, particularly in the context of sustainability. Still, these beneficial fungi are rarely implemented in large-scale agriculture. Hesitance is observed about the affectivity of AM fungi and the economic advantage of using these beneficial fungi. Results of applied AM fungi can become more convincing when environmental factors are taken into account.

2.1 Environmental Factors

Thus far, a large number of studies on mycorrhizal fungi have been carried out in which a wide variety of aspects were addressed, particularly in the last decades when molecular techniques have been developed. Among the aspects that have been studied and are still under investigation are genetics, physiology, ecology and application of mycorrhizal fungi. The majority of these studies focused on the mycorrhizal (Fig. 1) fungi and this has resulted in a great increase of knowledge. By centralizing mycorrhizal fungi in research projects (Fig. 1), we have gained insight in the fungi themselves, but we paid relatively little attention to the environment. In fact, the environment has considerable effects on the development and vitality of mycorrhizal fungi, an aspect that often has been overlooked. I would like to hold a plea to have more focus on the environment,

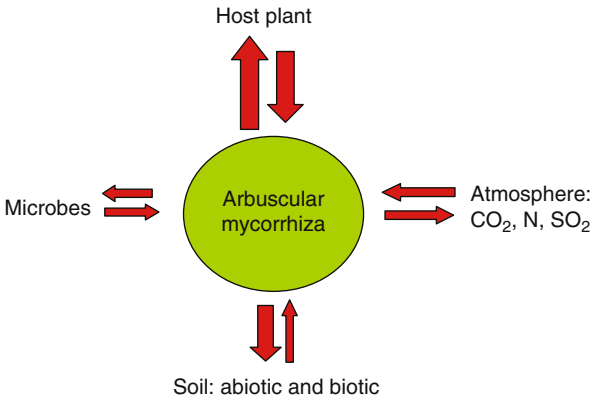

Fig. 1 Approach in which AM fungi are centralized with examples of environmental factors

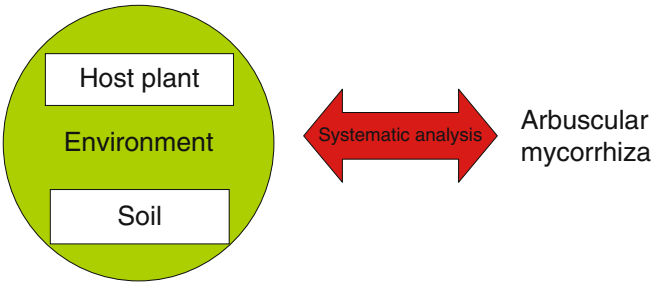

Fig. 2 Approach with a view from the environment with the main factors host plant and soil

because of its severe effects on the development and vitality of mycorrhizal fungi. In fact, I would like to propose considering an environmental approach in mycorrhizal research and applied settings.

In such an approach, environmental factors are centralized (Fig. 2). One of the most important environmental factors is the plant. The plant, often indicated with host plant, is the essential part of the mycorrhizal symbiosis beside the fungus. In fact, the symbiosis is as much dependant on the host plant as on the fungus (Fig. 2).

Approaching mycorrhizal symbiosis from the plant, the plant considerably influences the effectiveness of the symbiosis by its susceptibility. In various experiments, it has been shown that plant species vary greatly in their responsiveness to mycorrhizal fungi (Streitwolf-Engel et al. 1997; Van der Heijden et al. 1998a, 1998b). Also, crops can vary in their mycorrhizal responsiveness as has been reported for a variety of crops (Ryan and Graham 2002).

Another important environmental factor that influences the development of mycorrhizal fungi and symbiosis is the soil (Fig. 2). In fact, the soil is the environment where the hyphen and mycelia of the mycorrhizal fungi continuously appear. The most predominant soil factors are the physical and chemical soil composition that directly affects the development and vitality of the mycorrhizal fungi. Still, it is amazing how little systematic research has been done to describe the effects of abiotic soil variables on the development of AM fungi as pointed out by Estaún et al. (2002). Besides a limited number of studies to the effects of phosphate in the soil on the development of AM fungi, other soil components have received relatively little study.

3 Plants as Part of the Mycorrhizal Symbiosis

The major main crops that are grown in the world are AM fungal plants. A good example is onion (*Allium cepa* L.). This crop that can associate with AM fungi is one of the leading vegetable crops worldwide and grown in more than 100 countries. The global distribution of onions is due to the universal acceptance for food and condiment, but certainly not due to its simplicity of growing. In fact, onion is a crop that is difficult to grow, and one of the major challenges is to provide onion plants with sufficient nutrients (Brewster 1994). The

Fig. 3 Low-input agricultural field in The Netherlands where onions are grown

root systems of onion consist of superficial roots that are rarely branched and lack root hairs and so there is a very inefficient uptake capacity of water and nutrients such as phosphate (Wininger et al. 2003). In fact, a massive amount of chemical fertilizer is needed to grow onions, the sustainability of which is highly questioned.

A big step forward would be the growth of major main crops, including onion, in more low-input systems with reduced fertilizer inputs (Fig. 3). In a greenhouse experiment described by De Melo (2003), application of AM fungi to onion plants without fertilizer input and no use of fungicides resulted in increased above- and belowground biomass between 50 and 60% and significant increase of soil rooting area. This suggests that the addition of AM fungi to major cropping systems could be of great benefit to sustainability.

However, poor responses of AM fungi to cultivars of the major main crops of economic value have been reported. Examples are corn (*Zea mays*), oats (*Avena sativa*), barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) (Koide et al. 1988; Hetrick et al. 1993; Kaeppler et al. 2000; Hess et al. 2005). Breeding programmes for agricultural plants have produced varieties or cultivars with a range of genetic differences (Estaún et al. 2002). Variation in mycorrhizal dependency of *Citrus* rootstocks to AM fungi was found in a study by Camprubi and Calvet (1996).

Breeding of crops had the aim to raise resistance to fungal and bacterial pathogens, but has resulted in suppression of AM fungal colonization and responsiveness (Toth et al. 1990). As a consequence, incompatible reactions between host plants and certain AM fungi may occur. Hetrick et al. (1993) noted that breeding of wheat cultivars may have resulted in high dependency on fertilizers and non-responsiveness to AM fungal colonization.

As in many biological processes, numerous genes could be involved in the symbiosis between host plant and mycorrhizal fungus. Experiments with different mycorrhiza-defective plant mutants indicate that root colonization of AM fungi is controlled by a large number of genes (Gollotte et al. 2002). The approach taken by most researchers focused on the identification of plant genes controlling essential steps in the symbiosis between host plant and AM fungus (Balestrini and Lanfranco 2006). A number of the mycorrhiza-regulated genes have been identified by this approach, but genes involved in the mycorrhization process are still unknown (Gollotte et al. 2002; Grunwald et al. 2004).

An additional approach could be to set up breeding programmes. Evidence is growing that wild accessions or old varieties of cultivars show more susceptibility to AM fungi than modern cultivars of these species, indicating that mycorrhizal responsiveness may have been bred out of some crops (Kapulnik and Kushnir 1991). Studies like this demonstrate that genetic traits determining mycorrhizal responsiveness exist in both the plants and the fungi involved in this symbiosis. This indicates the clear need of combining the knowledge of plant geneticists and breeders with scientists working on AM fungi in order to develop an understanding of the genetic basis for mycorrhizal responsiveness.

Cooperation between plant geneticists, breeders and mycorrhizal researchers is certainly warranted to develop optimal combinations of mycorrhizal responsiveness of crop varieties with the most beneficial AM fungi. Such cooperation is necessary because it is unlikely that the identification of the genetic basis for mycorrhizal responsiveness in plants will lead to the responsiveness with all species of AM fungi and their varieties.

Further studies are needed to unravel the genetic traits in the diverse crops that are of economic value worldwide in order to optimize mycorrhization resulting in higher yields and reduced development of plant pathogens in low input systems. Thus far, several studies on plant breeding have started to study the genetic basis for mycorrhizal responsiveness in crops. More studies on different crops are needed.

3.1 Soil as an Environmental Factor Affecting Mycorrhizal Symbiosis

Another important determining factor for the development of AM fungi is the soil. Soil consists of combination of solids, water-solutions and gases. The solids comprise rocks and minerals, weathered rock materials, organic matter and living organisms, like soil microbes and soil fauna. The gas phase in soil contains usually oxygen available to soil organisms and plant roots. In the soil, horizons can be distinguished. These horizons are formed by a combination of physical, chemical, biological and anthropogenic processes and activities.

The vast majority of soils in the world contain AM fungi, but the diversity and abundance can vary. When undisturbed soil is brought into cultivation, the community

of AM fungi usually changes. Two key factors drive such modification: change in the plant community from natural vegetation to a monocultural cropping system, and soil chemistry changing from a nutrient-poor system to a nutrient-enriched system.

Some studies show that soil mineral content and structure can affect AM fungal communities (Johnson et al. 1992; Oehl et al. 2003, 2005). Johnson et al. (1992) showed that the occurrence of six AM fungal species was influenced by soil type. The study by Oehl (2005) revealed that the AM fungal communities changed with soil depth and that different AM fungal species were observed in different soil layers.

The studies carried out thus far show that the development of AM fungi and their effects on plant growth are greater in soils with low nutrient content, in particular with low phosphorus. It is well known that fertilization with phosphorus reduces development and root colonization of AM fungi, but the magnitude of the effect is strongly affected by the fungi studied, the host plant and other environmental conditions (Smith and Read 1997; Ryan and Graham 2002).

Moreover, application of mineral fertilizers is expected to reduce the diversity of mycorrhizal fungi in the soil. A study in California, USA, revealed that increase nitrogen concentrations in the soil resulted in reduced numbers of AM fungal spores. In addition, raised soil nitrogen concentrations resulted in a change of the species composition with an increase of species of the mycorrhizal genus *Glomus* (Egerton-Warburton and Allen 2000).

There are some studies on the effects of other soil factors on the development of AM fungi, but their number is limited. For instance, a relatively low amount of root colonization of AM fungi was found in roots of the grass *Deschampsia flexuosa*, a dominant plant species in the understory vegetation in *Pinus sylvestris* forests in The Netherlands. The pH of the mineral soil ranged from 3.2 to 4.0 while the pH of the litter and humus layers was even lower and varied from 2.9 to 3.6. Nitrogen and potassium concentrations of the litter and humus layers were relatively high, up to 13.3 mg/kg and 15.4 mg/kg, respectively (Baar 1995; Baar and ter Braak 1996).

However, systematic studies in field settings, in which several soil parameters are related to colonization levels and diversity of AM fungi, have hardly been carried out thus far (Estaún et al. 2002).

The climatic region is another factor influencing the development of mycorrhizal fungi. In certain regions in the world, characteristic climatic conditions include long, dry and hot summers, with scarce and erratic rainfall, while in winters torrential rainfall may occur like in the Mediterranean area.

A considerable amount of land in the Mediterranean regions is arid or semi-arid. Barrow (1991) estimated that 50% of Spain consists of such dry lands. In these regions, plants are exposed to considerable environmental stresses, including shortage of water resources for prolonged periods of the year. In fact, the factor most limiting to plant growth is water availability in semi-arid and arid environments. As a result of water limitation, plants access to phosphorous is difficult, and therefore can also be limiting (Gupta 1989). It is well known that AM fungi can ameliorate the effects of water stress in (semi-)arid ecosystems and enhance the uptake of

phosphorous (Smith and Read 1997; Jeffries et al. 2002). Furthermore, AM fungal inoculum can have stimulating effects on the development of crops in semi-arid regions. For instance, in a study in Israel, it was shown that inoculation of chive *Allium schoenoprasum*) with an AM fungus inoculum based on *Glomus intraradices* resulted in increased production of crops classed as export grade quality (Wininger et al. 2003).

The efficacy of AM fungal inoculum can differ between crop species in the Mediterranean area. For instance, in a study to investigate the effects of different AM fungi on *Citrus* cultivation in Turkey, differences in plant growth and percentage of mycorrhizal infection occurred with different AM fungal species (Ortas et al. 2002). On the other hand, AM fungal isolates that are effective in a given situation in the absence of water stress may not be affective under conditions of drought stress, which often occurs in the Mediterranean area.

Still, our knowledge about the effects of climatic conditions on mycorrhizal fungi is limited. The number of systematic studies in which the development and diversity of AM fungi is related to climatic conditions in combination with abiotic soil variables is poor. However, such insight is needed for the effectiveness of AM fungi when applied.

3.2 Applied Mycorrhizal Products in Relation to Environmental Factors

The number of new small to medium sized companies around the world producing inoculum of mycorrhizal fungi has increased over the last decade. Just in Europe, there are currently ten to twenty companies actively producing and distributing mycorrhizal products. This means that these producers see market opportunities for application of mycorrhizal fungi.

Companies have taken different approaches in their market approach, ranging from products with single AM fungi for specific markets, to mixed products for general markets. The mycorrhizal products contain granular substrates made from mixed materials such as peat, compost, vermiculite, perlite, sand, and/or expanded clay in which segments of colonized roots, spores, and filamentous networks are distributed (Dalpé and Monreal 2004). The roots, spores, and hyphal networks are very often not detectable, because of their microscopic sizes.

The mycorrhizal products usually consist of one to multiple AM species, most usually *Glomus* species. The most frequently used AMF species for commercial inoculum is typically *Glomus intraradices* Schenck & Smith. This species is well adapted to both in vivo and in vitro propagation, can colonize a large variety of host plants, survive to long-term storage, and is geographically distributed all over the world. These characteristics make the *G. intraradices* species an excellent candidate for commercial inoculum. Several other AM fungi belonging to *Glomus*, but also to *Gigaspora*, *Scutellospora*, and *Acaulospora* genera, are gradually used for commercial inoculum production (Dalpé and Monreal 2004). There are also

mycorrhizal products at the market in liquid form and these often contain only spores. In some products, mycorrhizal fungi and growth-stimulating bacteria are used for a potentially better inoculum for plant protection and production.

Mycorrhizal products can be applied in different ways. The most important is that the AM fungi are applied in the nearest vicinity of the seeds and roots of the plants as possible. For instance, it is possible to add a mycorrhizal product to the roots of each individual plant. Another possibility is mechanically application to arable land during sowing of the seeds. Specially designed agricultural machines can be used to inoculate the seed with a relatively high speed over a large area.

Different experiments have shown that mechanical application of commercial products with AM fungi can be effective. Several studies in The Netherlands showed that addition of a mix of AM fungi with *Glomus* species to onion and leeks resulted in increased root colonization of AM fungi (Baar, personal observation) (Fig. 3).

For the application of mycorrhizal products, it is of importance to consider the chemical and physical soil conditions. A basic requirement is that mycorrhizal fungi are applied to a soil environment where appropriate conditions for the beneficial fungi are present for optimal development.

However, mycorrhizal fungi from commercial production do not always give positive results after application. The low effectivity of the AM fungi is caused by poor development of the hyphen and the symbiosis with the plants as can be observed by microscopic observations. A possibility is that the low affectivity is related to low susceptibility of the plants to which the mycorrhizal products were applied as has been discussed before in this chapter.

Another possibility is that the efficacy of the mycorrhizal products is influenced by the soil type. The chemistry and physical conditions of different soil types differ considerably, like for instance the soil types of sand, clay and peat. A mycorrhizal product that is effective in sandy soil can be rather inactive in clay soil. Sandy soil is characterized by low nutrient availability, low pH and a rather loose structure of minerals while clay soil generally has a relatively high nutrient availability and pH, and a vast texture. In a recent experiment in The Netherlands, the affectivity of certain mycorrhizal mixes of commercial origin was rather low in clay soil while good results were obtained in sandy soils (Baar, personal observation).

Also, the affectivity of the commercial AM fungal products can be reduced by high fertilization levels with phosphate and nitrogen (Gosling et al. 2006). In some places in Europe, arable land is fertilized with mineral nitrogen, phosphate and potassium in concentrations exceeding 150 kg/ha. Under such conditions, mycorrhizal development is poor and it is unlikely that the few AM fungi present are active in nutrient uptake.

Another reason for low affectivity of mycorrhizal products could be caused by the inconsistent quality of the products. However, there are problems in bringing high quality and fit for purpose AM fungal products to target markets (Von Alten et al. 2002; Dalpé and Monreal 2004). Because AM fungi are obligate symbionts, the production of AM fungal inoculum of the best quality is still challenging.

However, there is an urgent need for better control over the production and storage of AM fungal inoculum before selling the products. Therefore, there is a desire for a quality control system for AM fungal products (Baar and Josten 2005). For such a system, there is a need to determine the quantity and quality of the mycorrhizal fungi present in the products. Currently, the quantity of AM fungi in inoculum is determined by the number of infection units using a MPN-estimation, the standardized most probable number estimation (Porter 1979; Daniels and Skipper 1984). However, the MPN-estimation is considered to provide a less constant and stable measure than that desired (Von Alten et al. 2002). Moreover, Dodd et al. (2000) have reported that the MPN methodology underestimates the inoculums potential of spores. Also, detection of contamination by plant pathogens is needed to ensure the quality of the AM fungal inoculum.

During the last decade, molecular techniques have been developed which enable the description of specific AM fungi in soils (Redecker 2000; Jacquot et al. 2000; Alkan et al. 2006). These molecular techniques allow qualitative and quantitative description of mycorrhizal fungi in soil and roots, including fungi that have been applied as inoculum. Such molecular tracing techniques are of crucial importance to determine the quality of mycorrhizal products. Also, the efficacy of the applied mycorrhizal fungi to colonies the plant roots can be determined by molecular tracing techniques. These techniques enable tracing of mycorrhizal fungi that have been applied. However, quantitative molecular tools have only been developed for a limited number of AM fungi (Alkan et al. 2004, 2006). Further development of molecular techniques for quantification of individual strains of AM fungi is desired for tracing the effectiveness of AM fungal inocula. Highly desirable are techniques enabling high throughput analysis of AM fungal DNA in soil and root samples. Thus, high throughput analysis systems have to be developed such that qualitative and quantitative description of AM fungi can be carried out.

4 Conclusions

The development of sustainable agricultural systems with low-input of mineral fertilizers and chemical pesticides is an issue that is gaining ground. In Europe, the EU stimulates sustainability in a wide range of fields including agriculture. Mycorrhizal fungi are key organisms that can be beneficial in the development of strong and healthy crops with reasonable yield in low-input systems.

For successful use of the mycorrhizal fungi, the requirements are that the environmental conditions are such that these beneficial fungi are able to develop (Fig. 2). The plants have to respond to the mycorrhizal fungi, and the soil conditions must be suitable for the applied fungi.

There is a need for more varieties of main crops grown worldwide that are better responsive compared to the large number of varieties, hardly susceptible for AM

fungi that are currently used. Particularly, the development of mycorrhizal susceptible varieties of the major main crops grown world wide would be a big step forward. Therefore, breeding programmes have to be set up and plant breeders, geneticists, arable farmers and mycorrhizal researchers need to cooperate.

The other major issue addressed is that we start to increase our knowledge about the effects of soil conditions on the development of mycorrhizal fungi (Fig. 2). Especially, studies with systematic approaches are needed. Insight in the relation soil–mycorrhizal fungi will facilitate commercial producers of mycorrhizal fungi with their development for better fit-for-purpose inoculum that are effective.

In 2007, the EU-network COST Action 870 has started. The focus of this Action is to bring together scientists from different scientific fields ranging from plant breeding and plant genetics, (low-input) arable farming and applied mycorrhizal research. The synergism gained by combining the scientific areas of plant breeding and mycorrhizal research will be unique and will lead to new research breakthroughs that are needed for application purposes.

References

Alkan N, Gadkar V, Coburn J, Yarden O, Kapulink Y (2004) Quantification of the arbuscular mycorrhizal fungus *Glomus intraradices* in host tissue using real-time polymerase chain reaction. New Phytol 161:877–885

Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhizal fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Appl Environ Microbiol 72:4192–4199

Atkinson D, Baddeley JA, Goicoechea N, Green J, Sánchez-Díaz M, Watson CA (2002) Arbuscular mycorrhizal fungi in low input agriculture. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 211–222

Baar J (1995) Ectomycorrhizal fungi of Scots pine as affected by litter and humus. PhD dissertation, Agricultural University, Wageningen, The Netherlands

Baar J, Josten, E (2005) Application of mycorrhizal fungi: what is the customer interest? Programme and abstracts Cost 8.38 meeting, Wageningen, The Netherlands

Baar J, Ter Braak CJ (1996) Ectomycorrhizal sporocarp occurrence as affected by manipulation of litter and humus layers in Scots pine stands of different age. Appl Soil Ecol 4:61–73

Barrow CJ (1991) Land degradation. Cambridge University Press, Cambridge

Balestrini R, Lanfranco L (2007) Fungal and plant gene expression in arbuscular mycorrhizal symbiosis. Mycorrhiza 17:153–153

Brewster JL (1994) Onions and other vegetable alliums. In: J Atherton (ed) Crop production science in horticulture. CABI, Wallingford, p 236

Camprubi A, Calvet C (1996) A field inoculation system for citrus nurseries using pre-cropping with mycorrhizal aromatic plants. Fruits 51:133–137

Copley J (2000) Ecology goes underground. Nature 406:452–454

Dalpé Y, Monreal M (2004) Arbuscular mycorrhiza inoculum to support sustainable cropping systems. Crop Manage. Online

Daniels BA, Skipper HD (1984) Methods for the recovery and quantitative estimation of propagules from soil. In Schenck NC (ed) Methods and principles of mycorrhizal research, The American Phytopathological Society, St Paul, Minnesota, United States of America, pp 29–38

De Melo PE (2003) The root system of onion and *Allium fistulosum* in the context of organic farming: a breeding approach. PhD dissertation, Wageningen University, Wageningen, The Netherlands

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

Egerton-Warburton LM, Allen EB (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient Ecol Appl 10:484–496

Estaún V, Camprubi A, Joner EJ (2002) Selecting arbuscular mcyorrhizal fungi for field application. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel pp 249–259

Gollotte A, Brechenmacher L, Weidmann S, Franken and Gianinazzi-Pearson V (2002) Plant genes involved in arbuscular mycorrhiza formation andd functioning. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 87–102

Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agric Ecosyst Environ 113:17–35

Grunwald U, Nyamsuren O, Tamasloukht M.B, Lapopin L, Becker A, Mann P, Gianinazzi-Pearson V, Krajinski F, Franken P (2004) Identification of mycorrhiza-regulated genes with arbuscule development-related expression profile. Plant Mol Biol 55:553–566

Gupta RK (1989) Drought response in fungi and mycorrhizal plants. In: Arora DK, Rai B, Mukeriji KG, Knudsen GR (eds) Handbook of applied mycology, vol I: soil and plants. Dekker, New York, pp 55–76

Hess JL, Shiffler AK, Jolley VD (2005) Survey of mycorrhizal colonization in native, open-pollinated, and introduced hybrid maize in villages of Chiquimula, Guatemala. J Plant Nut 28:1843–1852

Hetrick BAD, Wilson GWT, Cox TS 1993. Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. Can J Bot 71:512–518

Jacquot E, Van Tuinen D, Gianinazzi S and Gianinazzi-Pearson V (2000) Monitoring species of arbuscular mycorrhizal fungi in planta and in soil by nested PCR: application to the study of the impact of sewage sludge. Plant Soil 226:179–188

Jeffries P, Craven-Griffiths A, Barea JM, Levy Y and Dodd JC (2002) Application of arbuscular mycorrhizal fungi in the revegetation of desertified Mediterranean ecosystems. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 151–174

Johnson NC, Tilman D, Wedin D (1992) Plant and soil controls on mycorrhizal fungal communities. Ecology 73:2034–2042

Kaeppler SM, Parke JL, Mueller SM, Senior L, Struber C, Tracy WF (2000) Variation among maize inbred lines and detection of quantitative trait loci for growth at low phosphorus and responsiveness to arbuscular mycorrhizal fungi. Crop Sci 40:358–364

Kapulnik Y, Kushnir U (1991) Growth dependency of wild, primitive and modern cultivated wheat lines on vesicular-arbuscular mycorrhiza fungi. Euphytica 56:27–36

Koide RT, Li M, Lewis J, Irby C (1988) Role of mycorrhizal infection on growth and reproduction of wild vs. cultivated plants. I. Wild vs. cultivated oats. Oecologia 77:537–542

Lammerts van Bueren ET (2002) Organic plant breeding and propagation: concepts and strategies. PhD dissertation, Wageningen University, Wageningen, The Netherlands

Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Appl Environ Microbiol 69:2816–2824

Oehl F, Sieverding E, Ineichen K, Ris E-A, Boller T, Wiemken A (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 2005:273–283

Ortas I, Ortakçi D, Kaya Z, Çinar A, Önelge N (2002) Mycorrhizal dependency of sour orange (*Citrus aurantium* L.) in term of phosphorus and zinc nutrition by different levels of phosphorus and zinc application. J Plant Nutr 25:1263–1279

Porter WM (1979) The most probable number method for enumerating infective propagules of vesicular arbuscular mycorrhizal fungi in soil. Aust J Soil Res 17:515–519

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Ryan MH, Graham JH (2002) Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil 244:263–271

Schimel J (1995) Ecosystem consequences of microbial diversity and community structure. In: Chapin FS, Korner C (eds) Arctic and alpine biodiversity: patterns, causes, and ecosystem consequences. Springer, Heidelberg, pp 239–254

Smith SE, Read D (1997) Mycorrhizal symbiosis. Academic, San Diego, Calif.

Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1997) Clonal growth traits of two *Prunella* species are determined by co-occurring arbuscular mycorrhizal fungi from a calcareous grassland. J Ecol 85:181–191.

Toth JD, Toth D, Starke D, Smith DR (1990) Vesicular-arbuscular mycorrhizal colonization of *Zea mays* affected by breeding for resistance to fungal pathogens. Can J Bot 68:1039–1044

Van Bruggen AHC (1995) Plant Disease severity in high-input compared to reduced-input and organic farming systems. Plant Dis 79:976–984

Van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998a) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998b) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Visser S, Parkinson D (1992) Soil biological criteria as indicators of soil quality: soil microorganisms. Am J Alt Agric 7:33–37

Von Alten H, Blal B, Dodd JC, Feldmann F, Vosatka M (2002) Quality control of arbuscular mycorrhizal inoculum in Europe. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 281–296

Vosatka M, Dodd JC (2002) Ecological considerations for successful application of arbuscular mycorrhizal fungal inoculum. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhäuser, Basel, pp 235–247

Wininger S, Gadkar V, Gamliel A, Skutselsky Y, Rabinowitch E, Manor H, Kapulnik Y (2003) Response of chive (*Allium schoenoprasum*) to AM fungal application following soil solarization under field conditions. Symbiosis 35:117–128

Agronomic Management of Indigenous Mycorrhizas

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1 Introduction

Many benefits that accrue to plants from their association with arbuscular mycorrhizal (AM) fungi are a function of the increased volume of soil that can be explored by the extraradical mycelium. Sieverding (1991) estimates that for each centimeter of colonized root there is an increase of 15 cm^3 on the volume of soil explored, this value can increase to 200 cm^3 depending on the environmental conditions. The enhanced volume of soil explored, together with the ability of the extraradical mycelium to absorb and translocate nutrients to the plant, results in one of the most obvious and important advantages of mycorrhizal formation: the ability to take up more nutrients. The more important nutrients in this respect are those that have limited mobility in soil, such as phosphorus (P). In addition to nutrient acquisition many other benefits are associated with AM plants (Gupta et al. 2000): alleviation of water stress (Augé 2004; Cho et al. 2006), protection from root pathogens (Graham 2001), tolerance to toxic metals (Audet and Charest 2006), tolerance to adverse temperature, salinity and pH (Sannazzaro et al. 2006; Yano and Takaki 2005), and better performance following transplantation shock (Subhan et al. 1998). The enhanced tolerance to toxic metals provide by arbuscular mycorrhizas can be of benefit in phytoremediation (Göhre and Paszkowski 2006). The extraradical hyphae also stabilize soil aggregates by both enmeshing soil particles (Miller and Jastrow 1990) and as a result of the production of substances that adhere soil particles together (Goss and Kay 2005).

Agricultural practices such as crop rotation, tillage, weed control and nutrient management all produce changes in the soil chemical, physical and biological properties, and can modify the ecological niches available for occupancy by the soil biota. All these changes can influence in various ways the performance of the symbiotic relationship between the higher plant and the AM fungi, the potential for the production of inoculum for new mycorrhizas, and consequently effecting changes

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in the balance of indigenous AM fungal communities. The molecular tools that have been developed in recent years have been very important in broadening our understanding of these changes, as well as building a greater awareness of the consequences, which the choice of management practice imposes on AM development. The interest in extensive farming systems, environmental sustainability and the economics of production focuses attention on the identification of agronomic management practices that may allow controlled manipulation of the fungal community and capitalize on the mutualistic effects possible through mycorrhizal formation. In addition, they encourage evaluation of the use of local AM fungal inoculum as an option for mycorrhiza promotion and development in sustainable crop production.

In this chapter, we review the opportunities and limitations to the exploitation of recent research on arbuscular mycorrhizas in the development of sustainable crop production systems. Key aspects of agricultural land management that are considered include the choice of cropping sequences, the need for tillage, maintenance of soil structure, soil fertility and crop nutrition, weed and disease control. In addition, the issues around the use of AM fungal inoculum are presented.

2 Use of AM Fungal Inoculum

Despite the ubiquitous distribution of mycorrhizal fungi (Smith and Read 1997), with only a relative specificity between host plants and fungal isolates (McGonigle and Fitter 1990), the obligate nature of the symbiosis implies the establishment of a plant propagation system, either under greenhouse conditions or *in vitro* laboratory propagation. These techniques result in high inoculum production costs, which still remains a serious problem since they are not competitive with production costs of phosphate fertilizers. Even though farmers understand the significance of sustainable agricultural systems, the reduction of P inputs by using AM fungal inocula alone cannot be justified except, perhaps, in the case of some highly specialised crops (Saito and Marumoto 2002). Nurseries, large input horticultural enterprises and non-agricultural applications, such as restoration of degraded or devegetated landscapes, are examples of operations where the use of commercial inoculum is current.

A number of factors contribute to uncertainty in the minds of potential users. These include the quality of commercially available products, especially the guarantee that these are pathogen-free, the conditions required for storage before application, the most effective application methods and what is the appropriate inoculum for the application. Furthermore, information provided by suppliers about an inoculum can be deceiving, given that total counts of spores or propagules may be given, but only a fraction may be effective for a particular plant or under specific soil conditions. There is a clear need for registration procedures that can stimulate the development of the mycorrhizal industry (Gianinazzi and Vosátka 2004).

Advances in ecology during the past decade have led to a much more detailed understanding of potentially adverse consequences of introducing species into a

new habitat, but there is little information available on the ecological consequences of inoculation with mycorrhizal fungi. Schwartz et al. (2006) recommend that a careful assessment should be made prior to inoculation that documents the need for inoculation and the likelihood of success, because the introduction of mycorrhizal fungi is not universally beneficial. In addition, there is inadequate knowledge of the basic biology and diversity of AM fungi (Abbott at al. 1995; Saito and Marumoto 2002).

Some on-farm inoculum production and application methods have been developed to allow farmers to produce locally adapted isolates and generate a taxonomically diverse inoculum (Mohandas et al. 2004; Douds et al. 2006). However, inocula produced this way are not readily processed for mechanical application in the field, and this is an obstacle to their utilization in large scale agriculture, especially for row crops. Moreover, it requires an additional mechanical operation, with the corresponding economic and soil compaction costs.

Although inoculation with AM fungi has potential significance for sustainable crop production, including environmental conservation, current knowledge and technologies limit the application for widespread use in many agricultural contexts.

3 Crop Rotation

Crop rotation is an agronomic practice with a long history that is still performed and retains its general beneficial aspects associated with maintenance or improvement of soil fertility, reduction in erosion potential and in the build-up of pests, spreading of workload, lowering the risks from inclement weather damage, a lesser reliance on agricultural chemicals, and a need for increased net profits.

Although most crops support the formation of arbuscular mycorrhizas, roots of plants belonging to the Chenopodiaceae or Brassicaceae do not form these symbiotic relationships. Furthermore, the use of such crops in rotations tends to lead to a reduction in mycorrhizal propagules. In contrast, the cultivation of mycorrhizal host crops increases AM fungi populations and maintains mycorrhizal activity in soil (Vestberg et al. 2005). As a result, there tends to be much greater spore densities present (Black and Tinker 1977; Karasawa et al. 2000) leading to improved colonization of the succeeding crop, which may be in the following season (Gavito and Miller 1998a; Miller 2000).

Arihara and Karasawa (2000) studied the effects of fallow and prior cultivation of sunflower, maize, soybean, potato, sugar beet and canola (rapeseed) on the AM colonization of a subsequent maize crop. They found that shoot weight and grain yield of maize were much greater in the plots following sunflower, maize, soybean and potato than those after canola, sugar beet or a fallow. The cultivation of a non-AM host such as sugar beet or canola, reduces the mycorrhizal propagules and consequent AM colonization, P uptake and shoot dry weight (Arihara and Karasawa 2000; Gollner et al. 2004) even with no alteration of the availability of P in the soil

induced by the previous crop (Karasawa et al. 2001). These results established that cultivation of a mycorrhizal crop in the previous season promoted AM formation on roots of the following maize crop, which in turn enhanced its P uptake and growth, and finally increased the grain yield. The positive effect of having an AM host as the preceding crop for the maize was partly due to differences in AM fungal inoculum density (Karasawa et al. 2002).

Reduction of AM fungal propagules can also be a significant consequence of bare-fallowing. Because AM fungi are strictly biotrophic, their survival depends on the presence of host plants. During a bare-fallow the absence of host plants may cause the viability of AM fungi to decline and this decline may be further exacerbated by freezing conditions (Kabir et al. 1997b). Harinikumar and Bagyraj (1988) reported a 40% and 13% decrease in AM fungi propagules after fallow and a non-mycorrhizal crop, respectively. The extent to which AM fungi communities can be restored is inversely proportional to the duration of a fallow and the extent to which the land is grazed at the time (Duponnois et al. 2001).

Despite the ability of the extraradical mycelia to remain infective, even if the soil is frozen over winter (Addy et al. 1997), it is important to maintain the level of AM fungal inoculum to maximize the benefits of AM fungi on the following crop. Maintaining plant cover is very important, whether with cover crops or with cold tolerant crops, such as winter wheat. The choice of cover crops should be guided by the same principles as used to select the crop species to be adopted in a rotation. Preference should be given to mycotrophic cover crops capable of surviving in less favorable conditions while maintaining the AM inoculum potential in soil. Kabir and Koide (2000) demonstrated that using wheat or dandelion as mycotrophic winter cover crops increased subsequent sweet corn yield. Kabir and Koide (2002) reported that, relative to fallow, oats and rye were equally effective as cover crops in increasing mycorrhizal colonization in a succeeding sweet corn crop, as determined by the density of mycorrhizal hyphae and soil aggregate stability. Their results also underlined the importance of host crop diversity as they found that the combination of two cover crops (rye and oats) was significantly better than sowing a single species for the colonization of AM fungi, P uptake and yield of the following sweet corn crop.

Even though there is no obvious specificity between a host plant and colonizing AM fungal species, there are preferential associations (McGonigle and Fitter 1990; Vandenkoornhuyse et al. 2002; Gollotte et al. 2004). Furthermore, the cultivated host species can influence the abundance of the different AM fungi species present (Troeh and Loynachan 2003). This explains how crop rotations can cause changes in a mycorrhizal fungal community (Johnson et al. 1991; Hendrix et al. 1995) and the stability of complex soil biotic communities (Cavagnaro et al. 2006), and increase biodiversity (Miranda et al. 2005). In contrast, monocultures tend to select specific AM fungi, which tend to be inferior mutualists (Johnson et al. 1992), making AM fungi a possible cause for the yield decline often observed when such cultures are grown over long periods of time.

Different host species and cultivars of the same species vary in the degree to which they form mycorrhizas (Azcón and Ocampo 1981). The level of AM colonization

of a cultivar, and its associated benefits, is a heritable trait that can be selected through plant breeding (Kesava et al. 1990). Breeding programs are commonly conducted on experimental stations where mineral nutrients are not limiting factors. However, as increasing soil fertility can impair mycorrhizal development; this can result in the selection of host–crop varieties that form mycorrhizas less readily. For example, Hetrick et al. (1993) reported that the wheat cultivars released prior to 1950 were consistently greater in their level of dependence on mycorrhizal formation for production relative to more recent releases. Zhu et al. (2001) showed that modern wheat cultivars were less responsive to mycorrhizal colonization than were historical lines. The breeding of maize for resistance to fungal pathogens has produced lines that are less mycotrophic than previous varieties (Toth et al. 1990).

4 Soil Aggregation and Tillage Regime

Arbuscular mycorrhizas make direct contributions to soil aggregation and aggregate stability (Tisdall and Oades 1982), especially in no-till systems where hyphal networks remain intact. The direct effect of AM hyphae on soil aggregate formation was shown by Thomas et al. (1993) to be significant and at least equivalent to that of roots alone.

Important aggregate cementing agents produced by soil fungi and bacteria have been characterized as extracellular polysaccharides (Tisdall 1991), although Wright and Upadhyaya (1996) reported the presence of copious amounts of glomalin, a glycoprotein, associated with AM fungi, later found to be located on the surface of active AM hyphae (Rillig et al. 2001), and that could be important for soil aggregate stability (Miller and Jastrow 1990). In a 3-year study, Wright et al. (1999) measured an annual increase in both aggregate stability and weight of glomalin in the top 5 cm of the soil in no-till plots compared with ploughed plots. They also found that when soil was collected from the grassland adjacent to the tillage experiment, the structure of the top 0–10 cm of the grassland soil was more stable than of the cultivated soil after several years under no-tillage and 4 years under conventional tillage. Although there was not a full biochemical characterization of glomalin, in their study, Driver et al. (2005) showed that the material was tightly bound within the hyphal wall of AM fungi rather then being a primary release or secretion into the growth medium. They argued that glomalin has a role in the living fungus and any functionality in the soil was only secondary, possibly due to its relative slow turnover rate in the environment (Steinberg and Rillig 2003; Driver et al. 2005; Goss and Kay 2005; Rillig and Mummey 2006).

Soil tillage serves many purposes, including weed control, preparation of the seed bed, and improved water capture and storage in the soil profile (Cook 1992). It is also used to incorporate fertilizer, manure and pesticides and to reduce the incidence of disease and pests. Unfortunately, through the gradual loss of organic matter from near the soil surface, tillage can reduce aggregate stability and make soil more vulnerable to wind and water erosion. The environmental impacts of soil erosion became very

evident in the 1930s in the USA, and since the 1950s, there has throughout the world been a gradual transition from the mouldboard plough to various forms of conservation tillage, including no-till, with minimum soil disturbance. The basic principles of no-till agriculture include sowing directly into soil using the special planting equipment that cuts through or displaces the covering of crop residues. Retaining residues on the soil surface reduces erosion, evaporation and limits weed growth. It also improves water infiltration through the enhanced activity of the soil biota and the maintenance of macro-porosity, even if there is some increase in bulk density (Lal et al. 2007). The transition to no-till has implications for environmental quality, particularly because of its effectiveness in reducing soil erosion and surface run-off, enhancing soil organic matter concentration near the soil surface, increasing soil biological activity and reducing the energy required for crop production.

The direct effects of the various tillage practices on fungi, particularly conventional tillage (CT) and no-tillage (NT) systems, are related to physical disruption of the hyphal network and to the mixing of surface residues within the soil profile, affecting the effectiveness of AM symbiosis in many ways (Kabir 2005).

When host plants are present and the soil is not disturbed, hyphae from colonized roots and mycelia network are the main source of inoculum; they colonize roots more rapidly and efficiently compared with spores (Read et al. 1976; Martins and Read 1997). The latter are considered as "long-term" propagules (Kabir 2005), mainly because it would take longer for spores to germinate and make contact with roots compared with colonization by runner hyphae from a well developed extraradical mycelium (Klironomos and Hart 2002).

Since the 1980s, many studies under field conditions or in pots have been developed, most of them using maize as host plant, to evaluate the effect of soil disturbance on AM colonization and its consequences, particularly for P uptake. In one of the first reports on the subject, Evans and Miller (1988) described a significant adverse effect of soil disturbance on AM colonisation of both maize and wheat roots (both mycorrhizal), and also upon the P absorption by these species but no effects were found with respect to spinach and canola comparisons (nonmycorrhizal). Moreover, the injection of benomyl, a potent inhibitor of mycorrhizal fungi, into the soil surface significantly reduced the influence of soil disturbance on P absorption. These results indicate that the negative effect of disturbance on P uptake is likely due to impaired AM associations. Later, Evans and Miller (1990) demonstrated that disruption of the hyphal network was directly responsible for much of the effect of soil disturbance on mycorrhizal colonization. Besides, deep ploughing (to more than 15 cm) hinders subsequent mycorrhiza formation by reducing propagules density in the rooting zone (Kabir et al. 1998b). Abbott and Robson (1991) found that under no-till there were more spores in the top 8 cm of soil whereas tilled soils had more spores in the 8–15 cm depth.

Under field conditions, Brito et al. (2006b) found greater AM root colonization of wheat and triticale cultivated under NT compared to CT plots. Goss and de Varennes (2002), Antunes et al. (2006b) and Brito et al. (2006b) used the same technique for disturbing the soil in pot experiments, and, consistently found greater AM colonization of soybean or wheat, growing in pots of undisturbed soil.

The faster AM colonization observed in undisturbed or no-tillage systems supports earlier uptake of P (Vivekanandan and Fixen 1991; McGonigle and Miller 1996b; McGonigle et al. 1999), conferring a comparative advantage to the crop in the initial growth period, independently of soil P content (Fairchild and Miller 1990; McGonigle and Miller 1996b) although not always (Vivekanandan and Fixen 1991).

The number of spores, length of extraradical mycelium and hyphal density, particularly in the row zone (Kabir et al. 1998a), have been found to be enhanced when soil disturbance is reduced (McGonigle and Miller 1996a; Boddington and Dodd 2000a; Galvez et al. 2001; Borie et al. 2006). In addition to the enhanced concentration of P in the plant there are similar effects on Zn and Cu concentrations (Kabir et al. 1998a), which is not surprising considering the metabolic activity of hyphae in this system is greater (Kabir et al. 1997a). However, plant concentrations of other more mobile elements, such as K, Ca or Mg did not change with tillage regime (Kabir et al. 1998a).

The ability of an AM fungus to promote growth in undisturbed soil is related to the spread of mycelium in the soil and the capacity of nutrient transfer to the roots and, in addition, a capacity for persistence and retention of functional capacity of the extraradical mycelium from one plant generation to the next (McGonigle et al. 2003) and this is why the survival of AM extraradical mycelium is particularly important, over winter in cold climates or over summer in Mediterranean climates, where field crop production is restricted to a few months. In Canada, Kabir et al. (1997b) evaluated the timing of tillage on the survival of AM fungal hyphae, either connected to or detached from corn roots and whether the extraradical mycelium was intact or disrupted. They verified that fall tillage severely reduced AM hyphae viability, whereas spring tillage had little effect. They also found that attachment or proximity to roots favoured over winter survival, but disruption of the extraradical hyphae far outweighed the benefits of host root presence on survival.

Given that there is no such thing as a fungal effect or a plant effect, but a cross effect of both symbionts, the extent of colonization in soil under different levels of soil disturbance is also influenced by the host plant. Under the same experimental conditions, Mozafar et al. (2000) found an increase in AM colonization of maize under no-till, although there were no differences in colonization between tillage treatments in AM wheat.

Some exceptions to the promoting effects of no-tillage systems on AM colonization have also been reported. On one hand, Gavito and Miller (1998a) did not find any effect of tillage practices or fertilizer application, on the AM fungal colonization of maize under field conditions. On the other hand, even though Mozafar et al. (2000) observed greater AM colonization in maize under NT system then under CT, they did not see differences between tillage systems when wheat was the host crop. In other cases, despite the greater AM colonization and P content of plants cultivated under no-till than in conventional cultivation systems, it did not translate into enhanced growth yield (McGonigle and Miller 1996b; Galvez et al. 2001), suggesting an interaction of soil P and a yield depressing factor, possibly soil temperature, in no-till soils. In contrast to much previous research in mesic temperate climates,

Lekberg et al. (2008) found that in the semi-arid tropics, P fertilizer, long fallow periods, and tillage did not significantly decrease the AM fungi inoculum potential. McGonigle and Miller (2000) verified that the high inoculum density of particular ecosystems, such as the pastures studied in Australia, likely overrides any soil disturbance effect and ensures that roots of all plants became colonized by AM fungi.

An increased presence of AM fungi in roots from less disturbed systems has been reported to be accompanied by greater colonization by pathogenic fungi (McGonigle and Miller 1996a; Mozafar et al. 2000). The latter probably take advantage of the same mechanisms as AM fungi in terms of preserved integrity of the mycelium under these conditions. Mozafar et al. (2000) suggested that changes in nutrient concentration in the leaves of the plants tested in their study (wheat and maize) were likely due to the combined effects of colonization of the roots by various mycorrhizal and non-mycorrhizal fungi and not to changes in the chemical or physical properties of the soil. The authors stress the need to take into account nonmycorrhizal roots parasites and especially non-filamentous obligate fungi in studies conducted under field conditions.

4.1 Impact of Soil Disturbance on AM Fungal Communities

Colonizing strategies of AM fungi differ considerably and there is some indication that the variation is taxonomically based at the family level (Hart and Reader 2002). Consequently, different survival strategies for soil disturbance or tillage regime can affect the community of AM fungi of a particular site (Brito et al. 2006b).

Douds et al. (1995) found that *Glomus occultum* group was more numerous under NT, while the *Glomus* spp. and *Glomus etunicatum* groups were more numerous in soils under cultivation, even though spore characterization and quantification were done directly from field material. In the same study, field soil from the low input field plots produced greater colonization in a greenhouse assay than did soil from conventionally farmed plots. However, the host plant used, *Paspalum notatum* Flugge, differed from the ones in the field rotation (maize–soybean–wheat).

Other greenhouse experiments using inert attapulgite clay as a growth substrate and AM fungi from genera that differed in their sensitivity to soil disturbance, demonstrated that disturbance of preestablished extraradical mycelium reduced the formation of mycorrhizas by *Gigaspora rosea* but increased that by *Glomus manihotis* on *Desmodium ovalifolium* plants (Boddington and Dodd 2000b). Jansa et al. (2002) found a similar trend in their observations, which were based on morphological and molecular identification. There was an increase in the incidence of certain AM fungi, especially those not belonging to the *Glomus* spp., in intensively managed agricultural soil after long-term (13 years) reduced tillage. In these soils, an apparent increase in the incidence of *Gigaspora*, *Scutellospora* and *Entrophospora* was noted. In conventionally tilled soils, almost all the AM fungi present belonged to the genus *Glomus*. Surprisingly the authors did not find significant differences in AM fungi

diversity among different soil tillage treatments, even though the community structure was profoundly affected by the tillage treatment. Similar results were obtained by Franke-Snyder et al. (2001), who concluded that 15 consecutive years of farming with conventional or low-input agricultural management practices did not cause great differences among the fungal communities. The majority of the 15 fungal species found throughout the site were present in all treatments. The sporulation of a particular fungal species differed between farming systems or host plants but the general structure of AM fungal community was similar. These results contrast with those of Boddington and Dodd (2000a) and Oehl et al. (2003) who concluded that species richness can be reduced by soil disturbance.

Glomus spp. tends to survive perturbation and hence they prevail in highly disturbed agricultural systems, leading to the idea of a possible adaptation of different AM fungi species to different levels of soil disturbance. Oehl et al. (2003) also support the view that the result of this preferential selection favors the species that colonize roots slowly but form spores rapidly. AM fungi isolates from low input farming systems seem to be more efficient in promoting plant growth (Scullion et al. 1998; Oehl et al. 2004).

Jansa et al. (2003) made further progress using a nested PCR approach, and produced the first report on community composition of AM fungi in the roots of a field-grown crop plant (maize) as affected by soil tillage. Their results showed that the presence of the genus *Scutellospora* was strongly reduced in maize roots from ploughed and chiselled soils. Fungi from the suborder Glomineae were prevalent in roots from ploughed soils, but were also present in the roots from other tillage treatments. Jansa et al. (2003) attributed these changes in the community of AM fungi colonizing maize roots to differences in tolerance to the tillage-induced disruption of the hyphae between AM fungi species, together with changes in the nutrient content of the soil, microbial activity and changes in weed populations in response to tillage.

5 Weeds and AM Fungi

AM fungi colonize the roots not only of most agricultural crops but also of the weeds present (Yamato 2004). In most of the cases, AM fungi and weeds have co-evolved for a longer time than AM fungi and crops.

Agro-ecological functioning of weed communities may be affected by AM fungi through facilitative effects mediated by the mycelial network. Mycelial interconnections among host species in a weed-crop mixture may cause patterns of resource uptake and distribution among host species that differ qualitatively from those occurring in plant communities where AM fungi are absent (Moyer-Henry et al. 2006) as dying host species may release nutrients into the AM fungal mycelium (Smith and Read 1997), which may then be redistributed among other host species, enabling facilitative effects in crop–weed mixtures. For example, after selective weed control, nutrients acquired by host weeds may be transferred to host crop or

cover crop via the mycelium. Such processes may result in greater nutrient cycling and reduce competitive effects from nonhost weeds. If such phenomena occur and are qualitatively important, then AM fungi may be capable of significantly altering the agro-ecological functioning of weeds. Properly timed control operations, such as sublethal postemergence herbicide applications might be used to transfer nutrients from weeds to crops. In this scenario, the weeds might function as a temporary nutrient sink, restricting the competition for nutrients from nonhost weeds and reducing leaching and other mechanisms of nutrient loss.

Facilitative functions may also occur in which one host species supports populations of mycorrhizal fungi that are beneficial to another plant species. Host species may provide carbon to the mycelium which may support formation of arbuscular mycorrhizas with other species. In effect, the first host plants provide energy that serves, directly or indirectly, to support formation of AM colonization on a second newly germinating host. This allows the seedlings of the second host to receive nutrients and other mycorrhizal benefits while minimizing the energetic costs of mycorrhizal establishment to seedlings. For example, weed communities in several cropping systems have been shown to enhance mycorrhizal colonization and growth of subsequent crops, providing an alternative host between AM dependent crops (Kabir and Koide 2000; Brito et al. 2006a) or to maintain infective propagules over winter (Schreiner et al. 2001). The negative impact of fallow periods or the cultivation of nonhost crops on the AM inoculum may be mitigated by the presence of weeds in the field. Jansa et al. (2002) considered that the higher spore counts in no-till soil after the nonmycorrhizal plant (canola) was due to the increased presence of mycorrhizal weed plants in the no-till plots. These weeds may have supported AM fungal development in their roots and also allowed some spore formation under the canola. In the tilled plots, ploughing eliminated the majority of weeds and, therefore, AM fungi development during the growing of canola would be negligible. In Dehérain plots Plenchette (1989) also reported that when weeds were not controlled mycorrhizal infectivity was maintained. However, this effect would be beneficial only if the species that received the additional resources was desirable, and would have economic importance under the prevailing conditions: for example, under a Mediterranean climate, where the long, very dry summer might compromise the ability of extraradical mycelium to survive between the harvesting of one crop, in early summer, and the seeding of the next crop in the rotation, in autumn.

Feldmann and Boyle (1999) studied the interaction of weed competition and AM fungi in maize monoculture. They found decreased richness of AM fungal species and effectiveness in weed–free versus plots with weeds. Maize grew better in the presence of weeds and the authors concluded that the effective AM fungi overcompensate for any weed-mediated decrease in crop biomass. However, the benefit of enhanced AM fungi colonization of maize observed by Galvez et al. (2001) in the absence of effective weed control did not translate into enhanced growth or yield.

Although it is possible that AM fungi may have negative effects on agro-ecological functioning of weed communities, simply by increasing abundance of problematic weeds (Jordan et al. 2000), management of the existing weed population

might provide an important tool to guarantee a more rapid colonization of a winter crop with the consequent advantages of early adequate P nutrition. Abbott and Robson (1981) suggested that the many factors associated with the differences in effectiveness of different AM fungi in stimulating plant growth are due to their influence in the rapidity of infection rather than the ability of infected roots to take up P.

Given that colonization from spores is relatively slow because it requires a successful biochemical dialogue between plant roots and the AM fungal spores, the existence of weeds roots to initiate this process may be important so that fast colonization of the sown crop can take place from a well-established mycelium network as soon as germination occurs. In natural ecosystems or reduced-tillage agricultural systems, young seedlings can germinate and "plug" into an already established AM fungi hyphal network which permeates the soil and links different plant species. The lack of specificity confers a great advantage for the success of AM fungi in mixed plant communities. Another benefit to the plant of interest is that photosynthates are needed only for maintenance of the AM fungi following colonization, and not for the development of the extraradical mycelium since it was preestablished (Dodd et al. 2000).

Excessive tillage to control weeds, associated with frequent cultivation of non-mycorrhizal crops, could also hamper development of a diverse AM community (Gosling et al. 2006). In fact, Abbott and Robson (1991) reported a positive impact of weeds as host plants on the increase of AM fungi diversity. The multiplicity of roles that could be played by weeds of an agro-ecosystem in relation to arbuscular mycorrhiza formation requires careful planning of the timing and method for their control if the benefits are to be captured in terms of crop production.

6 Nutrient Management

The appropriate provision of macro- and micronutrients is a critical part of crop husbandry, to ensure both production potential and environmental safeguards are achieved. Investigation over the last two decades has indicated significant potential for the use of arbuscular mycorrhizas in enhanced nutrient management practices.

6.1 Phosphorus

In most agricultural systems, the application of P to the soil is necessary to ensure plant productivity. Phosphorus is largely taken up by plants from soil solution as inorganic PO_4^- ions. The recover of P applied as fertilizer or organic amendment by crop plants over a growing season is very small, as in the soil more then 80% of the P becomes immobilized and unavailable for plant uptake because of adsorption of ions on soil surfaces, precipitation of mineral phosphates or conversion to the

organic form (Holford 1997). Between 20 and 80% of soil P is present in organic form (Richardson 1994). The conversion of organic P into inorganic forms and the consequent availability to plants depends on hydrolysis either by micro organisms or by enzymes originated in the organisms themselves (autolysis). The remaining P is found in the inorganic (Pi) fraction, which may contain up to 170 mineral forms (Holford 1997). Pi may be held very firmly in crystal lattices of sparingly soluble forms, such as various Ca, Fe and Al phosphates, and may also be chemically bounded to the surface of clay minerals. This Pi exchanges very slowly with ions in the soil solution and constitutes a nonlabile pool, which is regarded as unavailable to plants. A small but less tightly bound P fraction exchanges relatively rapidly with the soil solution and constitutes a labile pool regarded as being available to plants.

Under conventional management practices, the role of the soil biota, including both saprophytic and mycorrhizal fungi, in nutrient cycling has been largely marginalized by the use of agrochemicals, particularly fertilizers but including fungicides, herbicides, and pesticides that counter disease and pests but can adversely affect other biota. Accumulation of P in the soil from applications of animal manure or fertilizer in excess of that taken up by the crop can increase the risk of P movement to surface and ground waters, with serious consequences for the aquatic environments. However, with increased societal pressures to reduce the use of agrochemicals and fertilizers, a greater reliance on processes influenced by soil biota, and especially AM fungi are assuming greater relevance. According to Grant et al. (2005), management of the cropping system to improve the availability of P to the crop early in the growing season may improve P nutrition while reducing the potential for excess accumulation of P in the soil and the risk of its transport into water systems. This requires a detailed understanding of the process governing soil P cycling and availability in which AM symbiosis may play a significant role.

Provision of adequate P early in crop development is usually directly related to an improved final grain yield (Gavito and Miller 1998b). For example, in maize, early P nutrition increased the number of kernels per plant, and similar increases in number or biomass of reproductive structures are reported for other crops (Gavito and Miller 1998b). The corollary is that deprivation of early P leads to a reduction in shoot growth accompanied by an initial stimulation of root growth (Mollier and Pellerin 1999).

An increase in the absorption of P by mycorrhizal plants can result by both the increased physical exploration of the soil and by increased transport into mycorrhizal fungus hyphae. Modifications of the rooting environment can enhance the transfer of P to plant roots and changes in the efficiency with which plants utilize P can all contribute to more effective crop nutrition (Bolan 1991).

Arbuscular mycorrhizal and non-AM plants show markedly different kinetics for P absorption, indicating that AM fungi hyphae have greater affinity for phosphate ions and a smaller threshold concentration for absorption than do plant roots (Smith and Read 1997). P is translocated in hyphae in the form of polyphosphates and translocation rates could be affected by concentration gradients and cytoplasmic streaming (Jakobsen 1992). Sanders and Tinker (1973) observed that the rate of inflow of P into AM roots was much greater than that of non-AM plants. By assuming that the

difference in the rate of inflow was all due to the AM fungi, they calculated that transport via mycorrhizal hyphae was six times that through a root hair.

Diffusion, rather then mass flow is the most important delivery system of P to plants, and movement through the soil is much slower than the rate of uptake into the roots. This results in a depletion zone around the roots (Smith and Read 1997). The size of the soil P depletion zone is significantly larger in AM plants then in non-AM plants. Li et al. (1991) found differences over 10 mm in some cases but in others this difference was greater than 110 mm, depending on the plant host colonized and the fungal isolate involved. In addition, they estimated that 80% of plant P could be supplied by AM fungi hyphae from as far as 100 mm distance beyond the zone of direct root exploitation. Bucher (2007) concluded from analysis of physiology, molecular and cell biology, and genetics of P uptake in vascular plants that soil P availability and the formation of P depletion zones around roots and their mycorrhizas are the major physical parameters determining plant P acquisition efficiency.

A wide range of biological events, and consequent environmental changes, occur in the rhizosphere and particularly in the mycorrhizosphere, leading to direct or indirect effects on the availability of sparingly soluble forms of P. The exudation of organic substances by roots and their formation and excretion by AM fungal hyphae might be important in facilitating host plant accessibility to available P or making P more available (Bolan 1991; George et al. 1995). Koide and Kabir (2000) showed that extraradical hyphae of *Glomus intraradices* can hydrolyze organic P and can transport the resultant inorganic P to host roots. However, direct evidence that organic acids such as oxalic and citric acid, which can make sparingly soluble P sources available, have only recently been reported for AM fungi. Tawaraya et al. (2006) developed a system that allowed them to collect exudates specifically from hyphae. Their results showed that hyphal exudates can actually contribute to increased P uptake into colonized roots. They found no oxalic acid production, and suggested that the type of organic acid produced might be related to the specific mycorrhiza and soil in which the fungi grew. They also stressed the importance of soil pH in relation to the ability of hyphal exudates to solubilise P. However, in a *in vitro* system, Antunes et al. (2007) do not fully confirm these results.

Other examples of mycorrhizosphere environmental modifications include the presence of P solubilizing companion microorganisms and changes in pH following AM colonization of roots. P is more readily available at pH 6.5, but for pH values above this the amount of sparingly soluble Ca-phosphates tend to increase, and for pH values less than 6.5 the levels of Al and Fe-phosphates increase.

In the rhizosphere, alkaline phosphatase activity is commonly greater than in the bulk soil, which contributes to increased availability of sparingly soluble P compounds. However, mycorrhizal hyphae do not appear to influence soil phosphatase activity, even where hyphal length density is considerable (Joner et al. 1995), but can influence alkaline phosphatase secretion by other microorganisms, probably through competition for nutrients (Joner and Jakobson 1995). In fact, the quantitative importance of extracellular phosphatases for P nutrition in AM plants, which has frequently been used to indicate promotion of P uptake by mycorrhizas, is considered to be insignificant relative to the total phosphatase activity in soil (Joner et al. 2000).

Jayachandran et al. (1989) suggested that siderophore production by AM fungi or other soil microbes could significantly increase P availability in low-pH soils, and that is a feasible mechanism by which AM plants could acquire P sources unavailable to non-AM plants.

P acquisition by AM plants varies not only with plant species and cultivar but also with the AM fungus colonizing the roots (Munkvold et al. 2004). The influence of the AM fungi species or isolate is effective at different levels: the size of the depletion zone, on the speed and rate of soil exploration by hyphae, phosphatase activity, the effectiveness of the symbiosis (as measured by the proportional AM benefit compared with the benefit from added P), P uptake by hyphae, shoot P content, growth performance at a given pH and accumulation of polyphosphates in the extraradical mycelium.

Fertilizer application, particularly P fertilizer and the associated increase in soil P level, decreases AM fungal infectivity and effectiveness (Dickson et al. 1999; Kahiluoto et al. 2000; Sorensen et al. 2005) and spore density (Chandrashekara et al. 1995). Irrespective of high levels of P in the soil, it is the concentration of P in the root system that determines whether AM fungi colonize roots (Menge et al. 1978). This suggests that the effect of P level in the soil on AM formation appears to be indirect, through the influence on P concentration in plant tissue, rather than directly on the fungus in the soil. This may explain why fertilizer application does not always reduce mycorrhizal associations. Fairchild and Miller (1988) observed extensive colonization at very high rates of fertilizer addition. Paradoxically, if the available P in the soil is very low, AM and spore production may be restricted and AM may increase by P application (Grant et al. 2005). For example, sunflowers grown in a sand medium that was almost P-free showed only poor mycorrhizal development, and the infection increased as P was added (Koide and Li 1990).

Addition of less soluble forms of P fertilizer, such as rock phosphate, effect P availability very slowly, and mycorrhizal colonization of the host plant is favored. The slow release of P likely prevents the P concentration in root tissue from reaching a level that can inhibit the formation of a mycorrhiza.

Also, the moderate application of farmyard manure seems to be less detrimental to root colonization by AM fungi than is the application of the same amount of nutrients in the form of inorganic NPK fertilizer (Joner et al. 2000). Gryndler et al. (2006) reported reduced growth of AM fungi (assessed by hyphal length and the signature fatty acid 16:1ω5) following mineral fertilizer application, but a manure application increased the growth of AM fungi. Colonization of roots followed the same pattern as the growth of AM fungi hyphae. In contrast, Allen et al. (2001) reported a greater AM colonization of dry bean and sweet corn in unamended than in manured soils.

Although no changes in AM fungi species composition were observed (Kahiluoto et al. 2001, 2004), cumulative P in soil decreased the size of the AM fungi communities, but did not affect the hyphal P transport capacity of hyphae (Kahiluoto 2004). Fertilizer application also seems to select for inferior AM mutualists (Johnson 1993).

Each combination of AM fungus, plant species or cultivar, and soil environmental condition is unique with several possibilities in terms of outcome for beneficial

effects on the plant and in terms of the uptake of P and other nutrients (Kahiluoto 2004; Jansa et al. 2005). However, the evidence for other nutrients is limited as they have been studied less. Pandey et al. (2005) found P uptake by wheat, rye and triticale was 10, 64 and 35%, respectively, greater in the presence of mycorrhizas than in their absence. The authors suggested that the differences in the enhancement of P uptake induced by mycorrhizal formation in triticale seem to be inherited from wheat rather than from rye. Different species of the same genera of the AM fungal symbionts, *Glomus mosseae* and G*lomus intraradices*, also exhibit a degree of functional complementary in terms of spatial P acquisition (Drew et al. 2003).

Burleigh et al. (2002) investigated whether functional diversity between AM fungi species is limited to the level of mycorrhizal formation, plant nutrient uptake and plant growth. Their study advanced current understanding of functional diversity and showed that plants can respond differently to AM fungi at the morphological and physiological levels and also at the level of gene expression. Functional diversity in mycorrhizal Pi uptake in different plant–AM fungi combinations and the control mechanisms involved are likely to be dependent on the molecular cross-talk between plant and fungal symbionts. Bucher (2007) suggested that to improve our knowledge of the role of fungal and plant metabolic status, elucidation of the chemical signals that orchestrate P transporter gene expression will possibly be the critical step towards a systems view of P uptake dynamics.

6.2 Nitrogen

In addition to the commonly reported enhanced P acquisition by AM plants, enhanced N acquisition is also often reported (George et al. 1995). Although it is thought that AM fungi have little ability to increase plant uptake of more mobile ions such as NO_3^-, as these move rapidly through the soil to roots, they do transport the less mobile NH_4^+ (Smith and Read 1997).

Corkidi et al. (2002) observed that enrichment with N consistently decreased root colonization by AM fungi in grasses grown in soils with high P availability, but not when they were grown under conditions of limited P availability. Such results point to the conclusion that when mycorrhizal costs exceed the benefits AM colonization is severely reduced, and are consistent with the hypothesis that N fertilizer application alters the balance between costs and benefits in mycorrhizal symbiosis. The authors also observed that AM fungal communities from N fertilized soils are less mutualistic than those from unfertilized soils.

Whether AM can take up or transform organic forms of N has long been a subject of debate, but recently Hodge et al. (2001) found that AM can enhance decomposition and increase N capture from complex organic material (grass leaves) in soil, independently of the host plant.

Considerable research has been conducted on the benefits conferred by the simultaneous AM colonization and nodulation of legumes with N_2-fixing bacteria. A synergistic effect between both microsymbionts and the host leguminous plant

lead to the idea of a tripartite symbiosis (El-Hassanin and Lynd 1985; Niemi and Eklund 1988) and has been explained on the basis of a high P demand in the N_2 fixation process, which is offset by the AM symbiosis (Stribley 1987; Smith and Read 1997). However, there is now evidence that P uptake is not the main driver for the development of the tripartite symbiosis. In fact, both symbioses share transduction pathways and effects of the tripartite symbiosis (e.g., increased nodulation) can be observed within 10 days after plant emergence, when the seedling still relies on P contained in the cotyledons (Goss and de Varennes 2002; Antunes et al. 2006a).

Variation in the enhancement of N acquisition by AM plants may also occur with different AM fungal isolates (Azcón-Aguilar et al. 1980). Antunes et al. (2006b) found no difference in N_2 fixation of soybean plants colonized with *Glomus clarum* or *Gigaspora margarita*. However, Chalk et al. (2006) highlighted the current paucity of quantitative data and lack of understanding of the interactions of legume genotype with AM fungal species in respect of the potential of AM to enhance legume yield and symbiotic dependence, particularly under field conditions.

Common mycorrhizal networks (He at al. 2003) can interconnect component intercropped species or cultivars by extending AM mycelia from one plant roots to another. The direct transfer of N from soybean to maize mediated through AM fungi has been shown by van Kessel et al. (1985) and between berseem and maize by Frey and Schüepp (1992). He et al. (2003) review not only one-way transfer of legume-N to a non N_2-fixing mycorrhizal plant but also a few studies showing transfer from nonfixing mycorrhizal plants to N_2-fixing mycorrhizal plants as a kind of bidirectional transfer. Hauggaard-Nielsen and Jensen (2005) believe that a better understanding of the mechanisms behind facilitative interactions may allow a greater benefit from these phenomena in agriculture and environmental management.

6.3 Other Nutrients

Clark and Zeto (2000) reviewed a number of studies where improved acquisition of sulphur (S) was observed in AM plants relative to uncolonized plants. Even though hyphae have not been shown to be highly active in S transport, the uptake of sulfur in AM plants appears to be very dependent on host plant, AM fungal symbiont, temperature and soil pH.

High boron (B) soil concentration may have negative effects on root AM colonization (Ortas and Akpinar 2006) and B acquisition in AM plants seems to be relatively inconsistent. It has been reported as enhanced (Kothari et al. 1990), reduced (Clark et al. 1999) and not affected (Lu and Miller 1989) in shoots of AM plants, and it is also reported that AM fungi differed extensively for B acquisition/restriction (Clark et al. 1999). The same kind of variation in nutrient acquisition, including the response to biotic and edaphic factors, has been noted for potassium, calcium, magnesium and sodium (Clark and Zeto 2000).

Uptake of both zinc (Zn) (Ryan and Angus 2003) and copper (Cu) are enhanced in AM plants but to a lesser extent than reported for P. These nutrients may not be as readily translocated from roots to shoots as is P, but the amount is probably adequate given that plants have a smaller requirement for these micronutrients. Its distribution in roots and shoots depends on soil P level. Zn, like P, is diffusion limited and its mobility in the soil is very small. AM involvement in Zn nutrition has been implicated from the negative effects of both tillage (Evans and Miller 1988) and long fallow (Wellings et al.1991) on crop growth. Beneficial responses to AM fungal colonization vary according to the different environmental conditions impacting the plants, especially soil pH.

AM plants generally acquire less manganese (Mn) than non-AM plants (Arines and Vilariño 1989; Bethlenfalvay and Franson 1989), although in acid soils, where Mn is more soluble, enhancement of Mn has been reported. Alleviation of Mn toxicity has also been reported frequently; it seems to be due to a more favorable equilibrium of Mn oxidising and reducing microorganisms in the mycorrhizosphere of AM plants (Nogueira et al. 2004).

Acquisition of iron (Fe) by AM plants is strongly affected by soil pH and the fungal symbiont. Amounts are reported to be enhanced or reduced depending on the conditions (Al-Karaki and Clark 1998; Nogueira and Cardoso 2002).

The presence of some elements also affects the way others are taken up in the presence of AM fungi. Liu et al. (2000) reported that, in the experimental conditions tested, the effect of AM fungi on Zn, Cu, Mn, and Fe uptake varied with micronutrient and P levels added to the soil.

For other potentially toxic elements (e.g., Cd, Pb, Ni, Ba, As), the ability of AM to grow in heavily contaminated sites has long been reported (Heggo et al. 1990; Ahmed et al. 2006). Apparently the toxic elements are sequestered in the hyphae, in the polyphosphate granules, and not transferred to the plant (Smith and Read 1997; Rivera-Becerril et al. 2002). Even when transferred, their negative influence on the plant metabolism is not critical because in AM plants the vegetative part is more developed allowing the dilution of trace elements. Different isolates have diverse tolerance to excess levels of many trace elements (Turnau et al. 2001). The size and diversity of AM fungi populations may be modified in metal-polluted soils (Del Val et al. 1999) and it appears that a prolonged exposure to this type of elements can result in the development of tolerance by the AM fungi (Oliveira et al. 2001).

7 Fungicides and AM Fungi

The use of pesticides, particularly fungicides, appears to impair mycorrhizal formation and development. In fact, most of the time the effects of these chemicals are detrimental to AM fungi (Manjunath and Bagyaraj 1984; Salem et al. 2003), although the degree of toxicity varies with the active ingredient, the application rate (Habte and Manjunath 1992) and the AM fungus (Schreiner and Bethlenfalvay

1997). Systemic fungicides would be expected to be more detrimental to AM fungi then nonsystemic ones. However, this distinction does not seem to be a key variable. For example, captan, a nonsytemic fungicide, can significantly reduce mycorrhizal formation while conversely triadimefon and pyrazophos, both systemic fungicides, actually promoted AM formation (von Alten et al. 1993). Kjøller and Rosendal (2000) concluded that external hyphae are more sensitive to the application of systemic fungicides, than are the internal hyphae.

An extensive review by Menge (1982) on the effects of many fumigants and fungicides specifically on AM fungi, is highly recommended despite the passage of time since it was published. A general analysis, confirmed by Vyas and Vyas (2000), indicates that for AM development, soil-applied products should be avoided.

8 Evaluation of AM Fungal Diversity

According to Douds and Millner (1999), an understanding of the impacts of agronomic practices upon communities of AM fungi would help to ensure an opportunity for the utilization of the symbiosis and contribute to the success of sustainable practices. Plenchette et al. (2005) argue that we need to be able to characterize AM fungi and mycorrhizas as easily as soil chemical properties, such as exchangeable cations. This approach could allow AM fungal characterization to be integrated into an agronomic diagnostic approach, and would help us establish when mycorrhizal development leads to poorer productivity. However, for the purpose of assessing the impact of any biotic or abiotic stress factor, measurement or evaluation of diversity in AM fungi communities in field soils, at least at the species level, presents a variety of challenges.

Spore characters and their various states of expression, especially spore wall structure (e.g., number of layers, size, color, refractivity, flexibility, histological reactivity, ornamentation) and developmental sequence with associated morphological features, have long been the basis for species-level taxonomy (Morton and Benny 1990). Identification and its appropriate application are further challenged by the obligate dependence of these fungi on plant host. Spores collected from the field lose or change the appearance of their structural characters in response to root pigments, soil chemistry, temperature, moisture and microbial activity. For the purpose of identification this necessitates the establishment of trap cultures to obtain healthy spores with clear structural characteristics. Depending on the set of plants chosen for the trap culture (Jansa et al. 2002), this technique may allow detection of nonsporulating members of the community. Mathimaran et al. (2005) have confirmed that the identity of plant species in trap pots significantly affected composition and diversity of associated AM fungi communities.

There is no clear relationship between functional diversity of organisms and the morphological diversity of spores used to delineate species (Douds and Millner 1999). Life-history traits that are important for the AM symbiosis (e.g., amount and

architecture of external hyphae, proportional fungal biomass as arbuscules versus vesicles and absorptive and transport capacity of hyphae) are not linked to any apparent character trait used to distinguish species (Morton and Bentivenga 1994). It is known that each AM fungal spore may have hundreds or thousands of nuclei (Becard and Pfeffer 1993), and although the genetic diversity has not yet been proven, it is possible to speculate that each individual carries the genome necessary to adapt to a wide variety of environmental conditions and plant hosts.

The morphology of fungal structures that can be detected in roots is in general very similar and does not necessarily reflect the extent to which different fungal species colonize the root systems. For a long time, the lack of reliable methods for the identification of fungal species that colonize a root greatly limited our ability to characterize changes in mycorrhizal populations (Miller et al. 1994). One of the most important objectives in AM research includes the need to find good and practical methods for describing communities. Serological (Hepper et al. 1988, Cordier et al. 1996), enzymatic (Hepper et al. 1986; Dodd et al. 1996), fatty acid profile (Graham et al. 1995) and many modern molecular tools have been developed and continuously improved in the last decade, and now constitute a powerful and promising approach to detect and evaluate this diversity, inclusively directly at soil level (van Tuinen et al. 2004).

9 Conclusion

Agricultural practices, such as tillage, crop rotation, crop residue retention and fertilizer use, all affect ecological niches available to the soil biota. Agro-ecosystems are managed biological systems that may involve the use of several practices that are detrimental to a mycorrhizal symbiosis. Furthermore, these systems also conform to the concept that organisms best adapted to the changing habitats, and niches will gradually replace those individuals not so well adapted (Rovira 1994). Conventional practices of land management usually result in a loss of the spatial heterogeneity of soil and diversity of organisms. In addition, it is very apparent that highly disturbed and unbalanced systems can became almost sterile habitats (e.g., mining sites) or completely dominated by only some groups of organisms (e.g., some weed species).

The goal of sustainable management is to create a more spatially heterogeneous habitat that offers the potential for more diverse and balanced systems to establish, in which natural occurring organisms, like AM fungi, can express their genetic potential, evolved over millions of years of co-existence with most terrestrial plants (Simon et al. 1993; Redecker et al. 2000). Management practices like no-till, controlled nutrient supply, and cautious control of weeds and pests, clearly have the possibility of encouraging arbuscular mycorrhizas.

Farmers have to be attentive and conscientious about the possible consequences of their management choices. There are no guaranteed “recipes” and each decision must take account of the multiplicity of economic and organizational aspects that

pertain locally, including production goals, constraints on the farm and the resources available. This new context is much more demanding for producers, since they must adapt our generalized understanding to their particular reality and be able to use the information for responsible and wise management decisions.

Communication between researchers and farmers is an essential element for success in developing new opportunities for a more effective use of mycorrhizal symbioses. Importantly, it must be strengthened by the flow of useful and easily understandable information in one direction, and clear, pertinent questioning and challenging in the other.

References

Abbott LK, Robson AD (1981) Infectivity and effectiveness of vesicular arbuscular mycorrhizal fungi: effect of inoculum type. Aust J Agric Res 32:631–639

Abbott LK, Robson AD (1991) Field management of VA mycorrhizal fungi. In: Keister DL, Gregan PB (eds) The rhizosphere and plant growth. Kluwer, Dordrecht, pp 355–362

Abbott LK, Robson AD, Scheltema MA, (1995) Managing soils to enhance mycorrhizal benefits in Mediterranean agriculture. Crit Rev Biotechnol 15:213–228

Addy HD, Miller MH, Peterson RL (1997) Infectivity of the propagules associated with extraradical mycelia of two AM fungi following winter freezing. New Phytol 135:745–753

Ahmed FRS, Killham K, Alexander I. (2006) Influences of arbuscular mycorrhizal fungus *Glomus mosseae* on growth and nutrition of lentil irrigated with arsenic contaminated water. Plant Soil 283:33–41

Al-Karaki GN, Clark, RB (1998) Growth, mineral acquisition, and water use by mycorrhizal wheat grown under water stress. J Plant Nutr 21:263–276

Allen BL, Jolley VD, Robbins CW, Freeborn LL (2001) Fallow versus wheat cropping of unamended and manure-amended soils related to mycorrhizal colonization, yield, and plant nutrition of dry bean and sweet corn. J Plant Nutr 24:921–943

Antunes PM, de Varennes A, Rajcan I, Goss MJ (2006a) Accumulation of specific flavonoids in soybean (*Glycine max* (L.) Merr.) as a function of the early tripartite symbiosis with arbuscular mycorrhizal fungi and *Bradyrhizobium japonicum* (Kirchner) Jordan. Soil Biol Biochem 38:1234–1242

Antunes PM, Deaville D, Goss MJ (2006b) Effect of two AMF life strategies on the tripartite symbiosis with *Bradyrhizobium japonicum* and soybean. Mycorrhiza 16:167–173

Antunes PM, Schneider K, Hillis D, Klironomos JN (2007) Can the arbuscular mycorrhizal fungus Glomus intraradices actively mobilize P from rock phosphates? Pedobiologia 51:281–286

Arihara J, Karasawa T (2000) Effect of previous crops on arbuscular mycorrhizal formation and growth of succeeding maize. Soil Sci Plant Nutr 46:43–51

Arines J, Vilariño A (1989) Use of nutrient: phosphorus ratios to evaluate the effects of vesicular-arbuscular mycorrhiza on nutrient uptake in unsterilized soils. Biol Fertil Soils 8:293–297

Audet P, Charest C (2006) Effects of AM colonization on "wild tobacco" plants grown in zinc-contaminated soil. Mycorrhiza 16:277–283

Augé RM (2004) Arbuscular mycorrhizae and soil/plant water relations. Can J Soil Sci 84:373–381

Azcón R, Ocampo JA (1981) Factors affecting the vesicular-arbuscular infection and mycorrhizal dependency of thirteen wheat cultivars. New Phytol 87:677–685

Azcón-Aguilar C, Barea JM, Olivares J (1980) Effects of Rhizobium polysaccharides on VA mycorrhiza formation. 2nd International Symposium on Microbial Ecology, University of Warwick, Coventry, U.K., Abstract No 187

Becard G, Pfeffer PE (1993) Status of nuclear division in arbuscular mycorrhizal fungi during in-vitro development. Protoplasma 174:62–68

Bethlenfalvay GJ, Franson RL (1989) Manganese toxicity alleviated by mycorrizae in soybean. J Plant Nutr 12:953–970

Black RLB, Tinker PB (1977) Interaction between effects of vesicular-arbuscular mycorrhiza and fertiliser phosphorus on yields of potatoes in the field. Nature 267:510–511

Boddington CL, Dodd JC (2000a) The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian ultisol. Plant Soil 218:137–144

Boddington CL, Dodd JC (2000b) The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. II. Studies in experimental microcosms. Plant Soil 218:145–157

Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil 134:189–207

Borie F, Rubio R, Rouanet JL, Morales A, Borie G, Rojas C (2006) Effects of tillage systems on soil characteristics, glomalin and mycorrhizal propagules in a Chilean Ultisol. Soil Tillage Res 88:253–261

Brito I, Carvalho M, van Tuinen D, Goss MJ (2006a) Effect of soil management and weed control on the diversity and establishment of arbuscular mycorrhizal colonisation of wheat in Mediterranean climates. In: Barea JM, Azcón C, Gutiérrez F, Gonzalez F, Molina AJ (Eds). 5th International Conference on Mycorrhiza. Estacion Experimental del Zaidín, Spain, p 268

Brito I, Carvalho M, van Tuinen D, Goss MJ (2006b) Effects of soil management on arbuscular mycorrhizal fungi in autumn-sown crops in Mediterranean climates. In: Horn R, Fleige H, Peth S, Peng X (eds) Soil management for sustainability. Advances in geoecology 38. Catena, Germany, pp 149–156

Bucher M (2007) Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol 173:11–26

Burleigh SH, Cavagnaro T, Jakobsen I (2002) Functional diversity of arbuscular mycorrhizas extends to the expression of plant genes involved in P nutrition. J Exp Bot 53:1593–1601

Cavagnaro TR, Jackson LE, Six J, Ferris H, Goyal S, Asami D, Scow KM (2006) Arbuscular mycorrhizas, microbial communities, nutrient availability, and soil aggregates in organic tomato production. Plant Soil 282:209–225

Chalk PM, Souza RD, Urquiaga S, Alves BJR, Boddey RM (2006) The role of arbuscular mycorrhiza in legume symbiotic performance. Soil Biol Biochem 38:2944–2951

Chandrashekara CP, Patil VC, Sreenivasa MN (1995) VA-mycorrhiza mediated P effect on growth and yield of sunflower (*Helianthus annuus* L.) at different P levels. Plant Soil 176:325–328

Cho K, Toler HD, Lee J, Ownley BH, Jean C. Stutz JC, Moore JL, Augé RM (2006) Mycorrhizal symbiosis and response of sorghum plants to combined drought and salinity stresses. J Plant Physiol 163: 517–528

Clark RB, Zeto SK (2000) Mineral acquisition by arbuscular mycorrhizal plants. J Plant Nutr 23:867–902

Clark RB, Zobel RW, Zeto SK (1999) Effects of mycorrhizal fungus isolates on mineral acquisition by *Panicum virgatum* in acidic soil. Mycorrhiza 19:167–176

Cook RJ (1992) Wheat root health management and environmental concern. Can J Plant Path 14:76–85

Cordier C, Gianinazzi-Pearson V, Gianinazzi S (1996) An immunological approach for the study of spatial relationships between mycorrhizal fungi in *planta*. In: Barea JM, Azcón-Aguilar C (eds) Mycorrhizas in integrated systems: from genes to plant development. European Commission, Luxembourg EUR 16 728:25–30

Corkidi L, Rowland DL, Johnson NC, Allen EB (2002) Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. Plant Soil 240:299–310

Del Val C, Barea J M, Azcón-Aguilar C (1999) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environ Microbiol 65:718–723

Dickson S, Smith SE, Smith FA (1999) Characterization of two arbuscular mycorrhizal fungi in symbiosis with *Allium porrum*. Colonization, plant growth and phosphate uptake. New Phytol 144:163–172

Dodd JC, Boddington CL, Rodriguez A, Gonzalez-Chavez C, Mansur I (2000) Mycelium of arbuscular mycorrhizal fungi (AMF) from different genera: form, function and detection. Plant Soil 226:131–151

Dodd JC, Rosendahl S, Giovannetti M, Broome A, Lanfranco L, Walker C (1996) Inter- and intraspecific variation within the morphologically similar arbuscular mycorrhizal fungi *Glomus mosseae* and *Glomus coronatum*. New Phytol 133:113–122

Douds Jr. DD, Millner PA (1999) Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. Agric. Ecosyst Environ 74:77–93

Douds Jr. DD, Galvez L, Janke RR, Wagoner P (1995) Effect of tillage and farming system upon populations and distribution of vesicular-arbuscular mycorrhizal fungi. Agric Ecosyst Environ 52:111–118

Douds Jr. DD, Nagahashi G, Pfeffer PE, Reider C, Kayser WM (2006) On-farm production of AM fungus inoculum in mixtures of compost-vermiculite. Bioresourc Technol 97:809–818

Drew EA, Murray RS, Smith SE, Jakobsen I (2003) Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. Plant Soil 251:105–114

Driver JD, Holben WE, Rillig MC (2005) Characterization of glomalin as a hyphal wall component of arbuscular mycorrhizal fungi. Soil Biol Biochem 37:101–106

Duponnois R, Plenchette C, Ba AM (2001) Growth stimulation of seventeen fallow leguminous plants inoculated with *Glomus aggregatum* in Senegal. Eur J Soil Biol 37:181–186

El-Hassanin AS, Lynd JQ (1985) Soil fertility effects with tripartite symbiosis for growth, nodulation and nitrogenase activity of *Vicia faba* L. J Plant Nutr 8:491–504

Evans DG, Miller MH (1988) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. I. Causal relations. New Phytol 110:67–74

Evans DG, Miller MH (1990) The role of the external mycelial network in the effect of soil disturbance upon vesicular-arbuscular mycorrhizal colonization of maize. New Phytol 114:65–71

Fairchild GL, Miller MH (1988) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. II. Development of the effect. New Phytol 110:75–84

Fairchild GL, Miller MH (1990) Vesicular-arbuscular mycorrhizas and the soil-disturbance-induced reduction of nutrient absorption in maize. New Phytol 114:641–650

Feldmann F, Boyle C (1999) Weed mediated stability of arbuscular mycorrhizal effectiveness in maize monocultures. Angew Bot 73:1–5

Franke-Snyder M, Douds Jr DD, Galvez L, Philips JG, Wagoner P, Drinkwater L, Morton JB (2001) Diversity of communities of arbuscular mycorrhizal (AM) fungi present in conventional versus low-input agricultural sites in eastern Pennsylvania, USA. Appl Soil Ecol 16:35–48

Frey B, Schuepp H (1992) Transfer of symbiotically fixed nitrogen from berseem (*Trifolium alexandrinum* L.) to maize via vesicular-arbuscular mycorrhizal hyphae. New Phytol 122:447–454

Galvez L, Douds Jr DD, Drinkwater LE, Wagoner P (2001) Effect of tillage and farming system upon VAM fungus populations and mycorrhizas and nutrient uptake of maize. Plant Soil 228:299–308

Gavito ME, Miller MH (1998a) Changes in mycorrhiza development in maize induced by crop management practices. Plant and Soil 198:185–192

Gavito ME, Miller MH (1998b) Early phosphorus nutrition, mycorrhizae development, dry matter partitioning and yield of maize. Plant Soil 199:177–186

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit Rev Biotechnol 15:257–270

Gianinazzi S, Vosátka M (2004) Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. Can J Bot 82: 1264–1271

Göhre V, Paszkowski U (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 223:1115–1122

Gollner M, Friedel JK, Freyer B (2004) Influence of different agricultural practices on the arbuscular mycorrhiza in organic farming systems. In: Baar J and Josten E (eds) Applied plant

research, WUR, Netherlands: role of mycorrhiza in sustainable land management. COST 8.38 Meeting, 26–27.02.2004, Vught, Netherlands
Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. Mycorrhiza 14:111–117
Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agric Ecosyst Environ 113:17–35
Goss MJ, de Varennes A (2002) Soil disturbance reduces the efficacy of mycorrhizal associations for early soybean growth and N2 fixation. Soil Biol Biochem 34:1167–1173
Goss MJ, Kay BD (2005) Soil aggregation. In: Wright SF, Zobel RW (eds) Roots and soil management: interactions between root and the soil. Agronomy Monograph No 48. ASA, CSSA, and SSSA, Madison, WI, USA, pp 163–180
Graham JH (2001) What do root pathogens see in mycorrhizas? New Phytol 149:357–359
Graham JH, Hodge NC, Morton JB (1995) Fatty acid methyl ester profiles for characterization ofglomalean fungi and their endomycorrhizae. Appl Environ Microbiol 61:58–64
Grant C, Bittman S, Montreal M, Plenchette C, Morel C (2005) Soil and fertilizer phosphorus: effects on plant P supply and mycorrhizal development. Can J Plant Sci 85:3–14
Gryndler M, Larsen J, Hrselova H, Rezacova V, Gryndlerova H, Kubat J (2006) Organic and mineral fertilization, respectively, increase and decrease the development of external mycelium of arbuscular mycorrhizal fungi in a long-term field experiment. Mycorrhiza 16:159–166
Gupta V, Satyanarayana T, Garg S (2000) General aspects of mycorrhiza. In: Mukherji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer, Dordrecht, pp 27–44
Habte M, Manjunath A (1992) Initial and residual toxicity of soil-applied thiram on the vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 2:25–31
Harinikumar KM, Bagyaraj DJ (1988) Effect of crop rotation on native vesicular arbuscular mycorrhizal propagules in soil. Plant Soil 110:77–80
Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344
Hauggaard-Nielsen H, Jensen ES (2005) Facilitative root interactions in intercrops. Plant Soil 274:237–250
He X, Critchley C, Bledsoe C (2003) Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). Crit Rev Plant Sci 22:531–567
Heggo A, Angle JS, Chaney RL (1990) Effects of vesicular- arbuscular mycorrhizal fungi on heavy metal uptake by soybeans. Soil Biol Biochem 22:865–869
Hendrix W, GuoB Z, An Z Q (1995) Divergence of mycorrhizal fungal communities in crop production systems. Plant Soil 170: 131–140
Hepper CM, Azcon-Aguilar C, Rosendahl S, Sen R (1988) Competition between three species of Glomus used as spatially separated introduced and indigenous mycorrhizal inocula for leek (*Allium porrum* L.). New Phytol 110:207–215
Hepper CM, Sen R, Maskall CS (1986) Identification of vesicular-arbuscular mycorrhizal fungi in roots of leek (*Allium porrum* L.) and maize (*Zea mays* L.) on the basis of enzyme mobility during polyacrylamide gel electrophoresis. New Phytol 102:529–539
Hetrick BAD, Wilson GWT, Cox TS (1993) Mycorrhizal dependence of modern wheat cultivars and ancestors: a synthesis. Can J Bot 71:512–518
Hodge A, Campbell CD, Fitter AH (2001) An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. Nature 413:297–299
Holford ICR (1997) Soil phosphorus: Its measurement, and its uptake by plants. Aust J Soil Res 35:227–239
Jakobsen I (1992) Phosphorus transport by external hyphae of vesicular-arbuscular mycorrhizas. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in ecosystems. CABI, Wallingford, UK, pp 48–54
Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jansa J, Mozafar A, Kuhn G, Anken T, Ruh R, Sanders IR, Frossard E (2003) Soil tillage affects the community structure of mycorrhizal fungi in maize roots. Ecol Appl 13:1164–1176

Jansa J, Mozafar A, Frossard E (2005) Phosphorus acquisition strategies within arbuscular mycorrhizal fungal community of a single field site. Plant Soil 276:163–176

Jayachandran K, Schwab AP, Hetrick BAD (1989) Mycorrhizal mediation of phosphorus availability: synthetic iron chelate effects on phosphorus solubilization. Soil Sci Soc Am J 53:1701–1706

Johnson, NC (1993) Can fertilization of soil select less mutualistic mycorrhizae? Ecol Appl 3:749–757

Johnson NC, Pfleger FL, Crookston RK, Simmons SR, Copeland PJ (1991) Vesicular-arbuscular mycorrhizas respond to corn and soybean cropping history. New Phytol 117: 657–663

Johnson NC, Copeland PJ, Crookston RK, Pfleger FL (1992) Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. Agron J 84:387–390

Joner EJ, Jakobsen I (1995) Growth and extracellular phosphatase activity of arbuscular mycorrhyzal hyphae as influenced by soil organic matter. Soil Biol Biochem 27:1153–1159

Joner EJ, Magid J, Gahoonia TS, Jakobsen I (1995) P depletion and activity of phosphatases in the rhizosphere of mycorrhizal and non-mycorrhizal cucumber (*Cucumis sativus* L.) Soil Biol Biochem 27:1145–1151

Joner EJ, van Aarle IM, Vosatka M (2000) Phosphatase activity of extra-radical arbuscular mycorrhizal hyphae: a review. Plant Soil 226:199–210

Jordan NR, Zhang J, Huerd S (2000) Arbuscular-mycorrhizal fungi: potential roles in weed management. Weed Res 40:397–410

Kabir Z (2005) Tillage or no-tillage: impact on mycorrhizae. Can J Plant Sci 85:23–29

Kabir Z, Koide RT (2000) The effect of dandelion or a cover crop on mycorrhiza inoculum potential, soil aggregation and yield of maize. Agric Ecosyst Environ 78:167–174

Kabir Z, Koide RT (2002) Effect of autumn and winter mycorrhizal cover crops on soil properties, nutrient uptake and yield of sweet corn in Pennsylvania, USA. Plant Soil 238:205–215

Kabir Z, O'Halloran IP, Fyles JW, Hamel C (1997a) Seasonal changes of arbuscular mycorrhizal fungi as affected by tillage practices and fertilization: hyphal density and mycorrhizal root colonization. Plant Soil 192:285–293

Kabir Z, O'Halloran IP, Hamel C (1997b) Overwinter survival of arbuscular mycorrhizal hyphae is favored by attachment to roots but diminished by disturbance. Mycorrhiza 7:197–200

Kabir Z, O'Halloran IP, Fyles JW, Hamel C (1998a) Dynamics of the mycorrhizal symbiosis of corn (*Zea mays* L.): effects of host physiology, tillage practice and fertilization on spatial distribution of extra-radical mycorrhizal hyphae in the field. Agric Ecosyst Environ 68:151–163

Kabir Z, O'Halloran IP, Widden P, Hamel C (1998b) Vertical distribution of arbuscular mycorrhizal fungi under corn (*Zea mays* L.) in no-till and conventional tillage systems. Mycorrhiza 8:53–55

Kahiluoto, H (2004) Systems management of AM in sustainable agriculture the case of P supply. In: Baar J and Josten E (Eds) Programme and abstracts of the COST-Meeting role of mycorrhiza in sustainable land management. Applied Plant Research, WUR, Netherlands

Kahiluoto H, Ketoja E, Vestberg M (2000) Promotion of utilization of arbuscular mycorrhiza through reduced P fertilization 1. Bioassays in a growth chamber. Plant Soil 227:191–206

Kahiluoto H, Ketoja E, Vestberg M, Saarela I (2001) Promotion of AM utilization through reduced P fertilization 2. Field studies. Plant Soil 231:65–79

Karasawa T, Arihara J, Kasahara, Y (2000) Effects of previous crops on arbuscular mycorrhizal formation and growth of maize under various soil moisture conditions. Soil Sci Plant Nutr 46:53–60

Karasawa T, Kasahara Y, Takebe M (2001) Variable response of growth and arbuscular mycorrhizal colonization of maize plants to preceding crops in various types of soils. Biol Fertil Soils 33:286–293

Karasawa T, Kasahara Y, Takebe M (2002) Differences in growth responses of maize to preceding cropping caused by fluctuation in the population of indigenous arbuscular mycorrhizal fungi. Soil Biol Biochem 34:851–857

Kesava RPS, Tilak KVBR, Arunachalam V (1990) Genetic variation for VA mycorrhiza-dependent phosphate mobilisation in groundnut (*Arachis hypogaea* L.). Plant Soil 122:137–142

Kjøller R, Rosendahl S (2000) Effects of fungicides on arbuscular mycorrhizal fungi: differential responses in alkaline phosphatase activity of external and internal hyphae. Biol Fertil Soils 31:361–365

Klironomos JN, Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181–184

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyze organic phosphate. New Phytol 148:511–517

Koide RT, Li M (1990) On host regulation of the vesicular-arbuscular mycorrhizal symbiosis. New Phytol 114:59–74

Kothari SK, Marschner H, Römheld V (1990) Direct and indirect effects of VA mycorrhizial fungi and rhizosphere microorganisms on acquisition of mineral nutrients by maize (*Zea mays*) in a calcareous soil. New Phytol 116:637–645

Lal R, Reicosky DC, Hanson JD (2007) Evolution of the plow over 10,000 years and the rationale for no-till farming. Soil Tillage Res 93: 1–12

Lekberg Y, Koid RT, Twomlow SJ (2008) Effect of agricultural management practices on arbuscular mycorrhizal fungal abundance in low-input cropping systems of southern Africa: a case study from Zimbabwe. Biol Fertil Soils DOI 10.1007/s00374-008-0274-6

Li XL, George E, Marchner H (1991) Extension of the phosphorus depletion zone in VA-mycorrhizal white clover in a calcareous soil. Plant Soil 136:41–48

Liu A, Hamel C, Hamilton RI, Ma BL, Smith DL (2000) Acquisition of Cu, Zn, Mn and Fe by mycorrhizal maize (*Zea mays* L.) grown in soil at different P and micronutrient levels. Mycorrhiza 9:331–336

Lu S, Miller MH (1989) The role of VA mycorrhizae in the. absorption of P and Zn by maize in field and growth. chamber experiments. Can J Soil Sci 69:97–109

Manjunath A, Bagyaraj DJ (1984) Effects of fungicides on mycorrhizal colonization and growth of onion. Plant Soil 80:147–150

Martins MA, Read DJ (1997) The effects of disturbance on the external mycelium of arbuscular mycorrhizal fungi on plant growth. Pesqui Agropecu Bras 32:1183–1189

Mathimaran N, Ruh R, Vullioud P, Frossard E, Jansa J (2005) *Glomus intraradices* dominates arbuscular mycorrhizal communities in a heavy textured agricultural soil. Mycorriza 16:61–66

McGonigle TP, Fitter AH (1990) Ecological specificity of vesicular-arbuscular mycorrhizal associations. Mycol Res 94:120–122

McGonigle TP, Miller MH (1996a) Development of fungi below ground in association with plants growing in disturbed and undisturbed soils. Soil Biol Biochem 28:263–269

McGonigle TP, Miller MH (1996b) Mycorrhizae phosphorus absorption and yield of maize in response to tillage. Soil Sci Soc Am J 60:1856–1861

McGonigle TP, Miller MH (2000) The inconsistent effect of soil disturbance on colonization of roots by arbuscular mycorrhizal fungi a test of the inoculum density hypothesis. Appl Soil Ecol 14:147–155

McGonigle TP, Miller MH, Young D (1999) Mycorrhizae crop growth and crop phosphorus nutrition in maize-soybean rotations given various tillage treatments. Plant Soil 210:33–42

McGonigle TP, Yano K, Shinhama T (2003) Mycorrhizal phosphorus enhancement of plants in undisturbed soil differs from phosphorus uptake stimulation by arbuscular mycorrhizae over non-mycorrhizal controls. Biol Fertil Soils 37:268–273

Menge JA (1982) Effect of soil fumigants and fungicides on vesicular-arbuscular fungi. Phytopathology 72:1125–1132

Menge JA, Steirle D, Bagy Araj DJ, Johnson ELV, Leonard RT (1978) Phosphorus concentrations in plants responsible for inhibition of mycorrhizal infection. New Phytol. 80:575–578

Miller MH, McGonigle T, Addy H (1994) An economic approach to evaluate the role of mycorrhizas in managed ecosystems. Plant Soil 159:27–35

Miller MH (2000) Arbuscular mycorrhizal and the phosphorus nutrition of maize: a review of Guelph studies. Can J Plant Sci 80:47–52

Miller RM, Jastrow JD (1990) Hierarchy of root and mycorrhizal fungal interactions with soil aggregation. Soil Biol Biochem 22:579–584

Miranda JCC, Vilela L, de Miranda LN (2005) Dynamics and contribution of arbuscular mycorrhiza in culture systems with crop rotation. Pesqui Agropecu Bras 40:1005–1014
Mohandas S, Gowda MJC, Manamohan M (2004) Popularization of arbuscular mycorrhizal (AM) inoculum production and application on-farm. Acta Hort (ISHS) 638:279–283
Mollier A, Pellerin S (1999) Maize root system growth and development as influenced by phosphorus deficiency. J Exp Bot 50:487–497
Morton J B, Benny G L (1990) Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. Mycotaxon 37:471–491
Morton JB, Bentivenga SP (1994) Levels of diversity in endomycorrhizal fungi (Glomales, Zygomycetes) and their role in defining taxonomic and nontaxonomic groups. Plant Soil 159: 47–59
Moyer-Henry KA, Burton JW, Israel D, Rufty T (2006) Nitrogen transfer between plants: A N-15 natural abundance study with crop and weed species. Plant Soil 282:7–20.
Mozafar A, Anken T, Ruh R, Frossard E (2000) Tillage intensity, mycorrhizal and nonmycorrhizal fungi, and nutrient concentrations in maize, wheat, and canola. Agron J 92:1117–1124
Munkvold L, Kjoller R, Vestberg M, Rosendahl S, Jakobsen I. (2004) High functional diversity within species of arbuscular mycorrhizal fungi. New Phythol 164:357–364
Niemi M, Eklund M (1988) Effect of VA mycorrhizae and bark ash on the growth and N2-fixation of two legumes. Symbiosis 6:167–180
Nogueira MA, Cardoso EJBN (2002) Interacções microbianas na disponibilidade e absorção de manganês por soja. Pesq Agropec Bras 37:1605–1612
Nogueira MA, Magalhães GC, Cardoso EJBN (2004) Manganese toxicity in mycorrhizal and phosphorus-fertilized soybean plants. J Plant Nutr 27:141–156
Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2003) Impact of land use intensity on the species diversity of arbuscular mycorrhizal fungi in agroecosystems of central Europe. Appl Environ Microbiol 69: 2816–2824
Oehl F, Sieverding E, Ineichen K, Mäder P, Boller T, Wiemken A (2004) Impact of conventional and organic farming on the diversity of arbuscular mycorrhizal fungi. In: Baar J and Josten E (eds) Programme and abstracts of the COST-Meeting role of mycorrhiza in sustainable land management. Applied Plant Research, WUR, Netherlands
Oliveira RS, Dodd JC, Castro PML (2001) The mycorrhizal status of phragmites australis in several polluted soils and sediments of an industrialised region of northern Portugal. Mycorrhiza 10:241–247
Ortas I, Akpinar C (2006) Response of kidney bean to arbuscular mycorrhizal inoculation mycorrhizal dependency in P and Zn deficient soils. Acta Agric Scan 56:101–109
Pandey R, Singh B, Nair TVR (2005) Impact of arbuscular-mycorrhizal fungi on phosphorus efficiency of wheat, rye, and triticale. J Plant Nutr 28:1867–1876
Plenchette C (1989) Potentiel infectieux mycorhizogene du sol des parcelles du dispositif Deherain. Rend Séances Acad Agric Fr 75:23–29
Plenchette C, Clermont-Dauphin C, Meynard JM, Fortin JA (2005) Managing arbuscular mycorrhizal fungi in cropping systems. Can J Plant Sci 85:31–40
Read DJ, Koveheri HK, Hodson J (1976) Vesicular-arbuscular mycorrhiza in natural vegetation systems. New Phytol 77:641–653
Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921
Richardson AE (1994) Soil microorganisms and phosphorus availability. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota: management in sustainable farming systems. CSIRO, Australia, pp 50–62
Rillig MC, Mummey, DL (2006) Mycorrhizas and soil structure. New Phytol 171:41–53
Rillig MC, Wright SF, Nichols KA, Schmidt WF, Torn MS (2001) Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. Plant Soil 233:167–177

Rivera-Becerril F, Calantzis C, Turnau K, Caussanel JP, Belimov AA, Gianinazzi S, Strasser RJ, Gianinazzi-Pearson V (2002) Cadmium accumulation and buffering of cadmium induced stress by arbuscular mycorrhiza in three *Pisum sativum* L. genotypes. J Exp Bot 53:1177–1185

Rovira AD (1994) The effect of farming practices on the soil biota. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota: management in sustainable farming systems. CSIRO, Australia, pp 81–87

Ryan MH, Angus JF, (2003) Arbuscular mycorrhizae in wheat and field pea crops on a low P soil: increased Zn_uptake but no increase in P-uptake or yield. Plant Soil 250:225–239

Saito M, Marumoto T (2002) Inoculation with arbuscular mycorrhizal fungi: the status quo in Japan and the future prospects. Plant Soil 244:273–279

Salem SF, Dobolyi C, Helyes L, Pçk, Z, Dimçny J (2003) Side-effect of benomyl and captan on arbuscular mycorrhiza formation in tomato. Acta Hort (ISHS) 613:243–246

Sanders FE, Tinker PB (1973) Phosphate flow into mycorrhizal roots. Pestic Sci 4: 385–395

Sannazzaro AI, Ruiz OA, Alberto EO, Menendez AB (2006) Alleviation of salt stress in *Lotus glaber* by *Glomus intraradices*. Plant Soil 285:279–287

Schreiner RP, Bethlenfalvay GJ (1997) Mycorrhizae, biocides, and biocontrol. 3. Effects of three different fungicides on developmental stages of three AM fungi. Biol Fertil Soils 24:18–26

Schreiner RP, Ivors KL, Pinkerton JN (2001) Soil solarization reduces arbuscular mycorrhizal fungi as a consequence of weed suppression. Mycorrhiza 11:273–277

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Scullion J, Eason WR, Scott EP (1998) The effectivity of arbuscular mycorrhizal fungi from high input conventional and organic grassland and grass-arable rotations. Plant Soil 204: 243–254

Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Deutsche Gesellschaft für Technische Zusammenarbeit (GTZ), Eschborn, Germany.

Simon L, Bousquet J, Lévesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London

Sorensen JN, Larsen J, Jakobsen I (2005) Mycorrhiza formation and nutrient concentration in leeks (*Allium porrum*) in relation to previous crop and cover crop management on high P soils. Plant Soil 273:101–114

Steinberg PD, Rillig MC (2003) Differential decomposition of arbuscular mycorrhizal fungal hyphae and glomalin. Soil Biol Biochem 35:191–194

Stribley DP (1987) Mineral nutrition. In: Safir GR (Ed) Ecophysiology of VA mycorrhizae plants. CRC, Boca Raton, Fla., pp 59–70

Subhan S, Sharmila P, Pardha Saradhi P (1998) *Glomus fasciculatum* alleviates transplantation shock of micropropagated *Sesbania sesban*. Plant Cell Rep 17:268–272

Tawaraya K, Naito M, Wagatsuma T (2006) Solubilization of insoluble inorganic phosphate by hyphal exudates of arbuscular mycorrhizal fungi. J Plant Nutr 29:657–665

Thomas RS, Franson RL, Bethlenfalvay GJ (1993) Separation of vesicular-arbuscular mycorrhizal fungus and root effects on soil aggregation. Soil Sci Soc Am J 57:77–81

Tisdall JM (1991) Fungal hyphae and structural stability of soil. Aust J Soil Res 29:729–743

Tisdall JM, Oades JM (1982) Organic matter and water-stable aggregates in soils. J Soil Sci 33:141–163

Toth R, Toth D, Stark D, Smith DR (1990) Vesicular-Abucular mycorrhizal colonization in *Zea mays* affected by breeding for resistance to fungal pathogens. Can J Bot 68: 1039–1044

Troeh ZI, Loynachan TE (2003) Endomycorrhizal fungal survival in continuous corn, soybean, and fallow. Agron J 95:224–230

Turnau K, Ryszka P, Gianinazzi-Pearson V, van Tuinen D (2001) Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 10:169–174

van Kessel C, Singleton PW, Hoben HJ (1985) Enhanced N-transfer from a soybean to maize by vesicular arbuscular mycorrhizal (VAM) fungi. Plant Physiol 79:562–563

van Tuinen D, Charvolin E, Gianinazzi-Pearson V (2004) Ribosomal sequences as a tool to study and monitor arbuscular mycorrhizal fungi. In: Baar J and Josten E (eds) Programme and abstracts of the COST - Meeting role of mycorrhiza in sustainable land management. Applied Plant Research, WUR, Netherlands

Vandenkoornhuyse P, Husband R, Fitter AH, Young JPW (2002) Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Mol Ecol 11: 1555–1564

Vestberg M, Saari K, Kukkonen S, Hurme T (2005) Mycotrophy of crops in rotation and soil amendment with peat influence the abundance and effectiveness of indigenous arbuscular mycorrhizal fungi in field soil. Mycorrhiza 15:447–458

Vivekanandan M, Fixen PE (1991) Cropping systems effects on mycorrhizal colonization, early growth, and phosphorus uptake of corn. Soil Sci Soc Am J 55:136–140

von Alten HA, Lindemann A, Schönbeck F (1993) Stimulation of vesicular-arbuscular mycorrhiza by fungicides and rhizosphere bacteria. Mycorrhiza 2: 167–173

Vyas SC, Vyas S (2000) Effect of agrochemicals on mycorrhizae. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer, New York pp 289–327

Wellings NP, Wearing AH, Thompson JP (1991) Vesicular-arbuscular mycorrhizae (VAM) improve phosphorus and zinc nutrition and growth of Pigeopea in a vertisol. Aust J Agric Res 42: 835–845

Wright SF, Upadhyaya A (1996) Extraction of an abundant and unusual protein from soil and comparison with hyphal protein of arbuscular mycorrhizal fungi. Soil Science 161:575–586

Wright SF, Starr JL, Paltineanu IC (1999) Changes in aggregate stability and concentration of glomalin during tillage management transition. Soil Sci Soc Am J 63:1825–1829

Yamato M (2004) Morphological types of arbuscular mycorrhizal fungi in roots of weeds on vacant land. Mycorrhiza 14:127–131

Yano K, Takaki M (2005) Mycorrhizal alleviation of acid soil stress in the sweet potato (Ipomoea batatas). Soil Biol and Biochem 37:1569–1572

Zhu YG, Smith SE, Barritt AR, Smith FA (2001) Phosphorus (P) efficiencies and mycorrhizal responsiveness of historical and modern wheat cultivars. Plant and Soil 237:249–255

AM Inoculation in Tropical Agriculture: Field Results

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1 Introduction

Tropical soils usually pose serious problems to plant production. It is considered that about 30% of tropical soils around the world have problems of nutritional stress and that only 8% have no stress at all for plant growing. The rest of the tropical soils may have problems of water stress, flooding or very poor development (Sieverding 1991).

The soil types most frequently found in tropical areas are Oxisols, Ultisols and Inceptisols. The two former occur in large areas from South America and Africa. Oxisols are the most highly weathered soils and they form in hot climates with nearly year-round moist condition. In consequence, the vegetation type that grows on them is generally tropical rain forest (Brady and Weil 2002). Their most important feature is the presence of a horizon very high in clay-size particles dominated by hydrous oxides of iron and aluminum. The clay content of Oxisols is generally high, but the clays are of low activity. The low-activity clays have a very limited capacity to hold basic cations, so they are typically of low natural fertility and moderately acid. The high concentration of iron and aluminum oxides also gives these soils a capacity to bind phosphorus tightly. So, phosphorus deficiency often limits plant growth. Millions of people in the tropics depend on Oxisols for food or fiber production (Brady and Weil 2002).

The majority of Ultisols have been developed under humid conditions in warm to tropical climates. Ultisols are formed on old land usually under forest vegetation although savannas are also common. They are characterized by a relatively acidic B horizon that has less than 35% of the exchange capacity satisfied with base cations. Certain Ultisols that were formed under fluctuating wetness conditions (e.g., savanna weather) have horizons of iron-rich mottled material called “plinthite”. If this material is dried in the air, plinthite hardens irreversibly into a kind of ironstone

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that is virtually useless for agriculture. Although Ultisols are not very fertile, they respond well to good management. Finally, Inceptisols, as their name indicates, are soils at the beginning of their development. They are often prominent in mountainous areas, especially in the tropics. Wet Inceptisols are found in areas along the Amazon and Ganges rivers.

It is evident, then, that tropical regions mostly have inherently low fertility. For this reason, the association between plants and fungi from the Glomeromycota Phylum to form arbuscular mycorrhizas (AM) could be an appropriate strategy when the sustainable management of tropical soils is the goal. Natural vegetation that thrives in acidic soils has ecophysiological mechanisms that let them survive and grow in such conditions (Cuenca et al. 2001). In contrast, many crop plants selected by man have lost these abilities or do not have them at all due to their particular origin. In consequence, the traditional management of acidic tropical soils has included the addition of chemical fertilizers (mainly as triple superphosphate) and liming in order to cultivate them.

In the last decade, there has been an attempt to change the former paradigms about crop production. Former practices involved the use of high inputs of energy, machinery and chemicals (the so-called "green revolution"). Today, the concept of sustainable agriculture is conquering new areas and followers. According to this new paradigm, the sustainable agriculture is "an integrated system of plant and animal production practices having a site-specific application that will, over the long-term: (1) satisfy human food and fiber needs; (2) enhance environmental quality and the natural resource base upon which the agricultural economy depends; (3) make the most efficient use of nonrenewable resources and onfarm resources and integrate, where appropriate, natural biological cycles and controls; (4) sustain the economic viability of farm operations; and (5) enhance the quality of life for farmers and society as a whole" (Jeffries and Barea 2001).

From that definition, it becomes evident that mycorrhizal symbioses could be a very useful tool for the achievement of a sustainable agriculture. In fact, there is an increasing public awareness about environmental quality, which is causing an increase in the interest for the so-called "organic" products, those that are produced without chemicals or agrochemicals (FIDA 2003). On the other hand, in neotropical areas there are still pristine waters which it is desirable to preserve, although in some places eutrophication problems are arising (Infante et al. 1979; González 2004). In several Latin American countries, adequate extension services are lacking and, in consequence, agrochemicals and chemical fertilizers are applied indiscriminately even by smallholders. It is important, then, to develop and divulgate new strategies of crop management that reduce these problems.

In a recent review, Cardoso and Kuyper (2006) emphasized the differences that there are between the management of soil fertility in tropical region versus temperate. For these and other authors (van Noordwijk and Cadish 2002), agriculture in temperate zones is often characterized by conditions of excess and that in tropical region by problems of access. Indeed, according to Cardoso and Kuyper (2006), in most tropical agro-ecosystems resource-poor farmers who spend money on buying mycorrhizal inoculum could have better spent that money on phosphorus fertilizers.

It is important to point out that, in many acidic tropical soils, the addition of P-fertilizer does not produce the inhibition of AM colonization (Cuenca et al. 2003;

Siquiera et al. 1998), in contrast to many reports in the literature (Smith and Read 1997). On the contrary, it is possible that, in the absence of fertilizer, the presence of mycorrhizas cause a growth depression due to the fact that carbohydrate cost cannot be compensated by the plant. Meanwhile, the fungus takes carbohydrates for its support, but no P is translocated to the plant due to its scarcity in soil (Cuenca et al. 1998). The use of rock phosphate has been proposed as an alternative to the use of synthetic fertilizer in view of its very large deposits in Africa and Latin America (Ramírez 2006).

Tropical areas are far from being homogeneous with high temperatures and humidity all the year. In fact, tropical countries include a variety of situations from dry to wet and flooding, and from hot temperatures to permanent frozen areas in the high tropical mountains. There are also a variety of situations for the farmers. There are several which associated the care of their crops with the use of high amounts of fertilizers, which in many cases are subsidized by the State. At the other extreme, there are indigenous farmers who depend on shifting cultivation that involves slashing and burning the forest, followed by continuous cultivation and then abandonment after 3–5 years because of low crop yield (Uhl 1987; Jordan 1987; Knapp et al. 1996).

In this chapter, we will present the common situation for agriculture in northern South America. We will show field results of traditional crop practices which can be contrasted with those that include a more ecological and sustainable management of resources.

Although there is a large bibliography regarding the benefits of AM in crop production, after the extraordinary work performed by Sieverding (1991) in Colombia, field results in tropical countries have been scarce. We will show some recent experiences about the use of AM fungal inocula in real conditions and, of course, in the presence of the indigenous AM fungi (AMF) populations.

2 Production Modes

2.1 Crops with a Seedbed Stage

Horticultural crops like lettuce are one of the most important items that need a seedbed stage in which AM inoculum can be introduced easily. In northern Venezuela, the imported variety of *Lactuca sativa* Great Lakes is sown and maintained in the seedbed for 45 days. Then, seedlings are transplanted to the field. If soil of seedbed is not fumigated or sterilized, the introduced AM inoculum should compete with indigenous AM fungi and, at the end of the seedbed stage, the effect of the introduced inoculum is lost (Table 1). If the seedbed is previously sterilized, the introduced AMF colonizes the roots of the host plant early on. Then, when the seedlings are transplanted to the field, there is a high probability that the introduced AMF will have become installed in the roots.

To save manpower, farmers cut the leaves and roots of lettuce seedlings before transplanting. They argue that with this practice time and money is saved because there is no necessity of manipulating roots to put them vertically in the planting

Table 1 Effect of AM-inoculation on the dry weight of *Lactuca sativa* in seedbed previously sterilized or not

	Dry weight (mg)	
Treatment	Non sterilized	Sterilized
Control	103.8 ab	40.7 a
Inoculated	106.4 ab	294.8 d
Fertilized	118.9 abc	90.7 ab
Inoculated + Fertilized	195.9 bcd	266.2 cd

Source: Oirdobro G. and Cuenca G., unpublished results. Means followed by the same letter are not significantly different, $p < 0.05$

Table 2 ANOVA probabilities of the effect of cutting leaves and roots, inoculation with *Glomus manihotis* and fertilization on the fresh weight of lettuce after 2 months in the field

Effect	*F*	*p* level
Cut (A)	2.31	0.13
Inoculation (B)	37.18*	0.00*
Fertilization (C)	19.06*	0.00*
A × B	0.74	0.39
A × C	1.51	0.23
B × C	4.80*	0.01*
A × B × C	0.46	0.63

Source: Oirdobro G. and Cuenca G., unpublished results.* Significant at 0.01 level

hole. This practice was evaluated together with the effect of AM inoculum and fertilizers that are normally applied by the small farmers in that region. The fertilizer dose normally applied by the farmers in the field (1,858 kg/ha of NPK) was compared with a reduced dose (180 kg/ha of NPK), suggested as being less aggressive for the environment, and consisting of a 90% reduction of the fertilizer dose.

To cut leaves and roots did not cause any significant effect in the fresh weight of lettuce (Table 2) as the farmer supposed, but a strong effect of both inoculation and fertilization was observed.

In the field, lettuce of similar (statistically) fresh weight were obtained substituting 90% of the chemical fertilizers by an inoculum of *Glomus manihotis* (Table 3). However, as was pointed out above, in acidic soils it is necessary to apply a certain dose of fertilizers to improve AM functioning as in this case.

2.2 Production in Nursery

Several of the most important tropical cultures such as coffee and cacao have a nursery stage where AMF can be introduced. Additionally, as in many areas of tropical America, these crops are sown under the shade of woody legume, e.g.,

Table 3 Shoot fresh weight of *Lactuca sativa* obtained after 2 months growing in the field inoculated with *Glomus manihotis* or not, and fertilized with two doses of NPK or not

Treatment	Fresh weight (g)
Control	28.7 a
F_H	102.0 b
F_L	321.4 bc
Inoculated	220.6 bc
F_H + inoculated	453.9 c
F_L + Inoculated	532.2 c

Source: Oirdobro G. and Cuenca G., unpublished results.
F_H = 1,858 kg/ha and F_L = 180 kg/ha of NPK (12:29:12) applied when transplanting to the field
Means followed by the same letter are not significantly different, $p < 0.05$

Erythrina or *Inga* spp. Both cultures can be considered as very suited ecologically as they function in a manner which is very similar to a simplified tropical forest. The mechanisms that preserves nutrient in the forest, like the decomposition of litter and the pumping of nutrients from the deepest layers of the soil profile up to surface layers that is performed by canopy trees, are both present in this very tropical and special kind of management. A recent tendency to improve the growth of coffee seedlings in nurseries has included the use of organic amendment like coffee pulp which is an abundant by-product obtained in postharvest (Osorio et al. 2002). Interestingly, the improvement of saplings growth achieved when AM were combined with coffee pulp virtually disappeared when it was chemically sterilized, demonstrating that the beneficial effects of the coffee pulp were due to the enhancement of soil microbial activity (Osorio et al. 2002).

The AMF spore distribution down the soil profile in shaded coffee cultures in comparison with monoculture coffee (unshaded) showed a greater number of spores in the deeper soil layers in shaded coffee systems. This may be explained by the greater quantity of roots, and may be an indicator of greater incidence of mycorrhizas in shaded than in monocultural coffee systems (Cardoso et al. 2003). In some tropical soils, the amount of P is not so low, but mainly present in unavailable forms (see Introduction). In such soils, greater mycorrhizal activity in the deeper soil layers may be important to make more P available to the plant, thus increasing the efficiency of nutrient recycling processes in these coffee systems (Cardoso et al. 2003).

Other important tropical crops like avocado can benefit from the application of AMF in the nursery (da Silveira et al. 2002). As is well known, not all AMF species perform in a similar manner, and a screening among the several inoculum available should be done to select the one that perform better in nursery conditions. *Scutellospora heterogama* and *Acaulospora scrobiculata* inocula produced the best results when tested with avocado *var* Carmen in a nursery in Brazil (da Silveira et al. 2002).

2.3 Smallholders Farming

In tropical mountains, farmers have the ownership of small areas, but they sow crops that produce high incomes due to their particular value. This kind of production system tends to use high amount of fertilizers and agrochemicals (Torres and Capote 2004). In some cases, farmers do not apply crop rotation, because they do not want lost their market place, so a number of soil pathogens and excess of chemicals accumulate in soils causing not only environmental problems but even affecting people health. In an attempt to reduce the chemical inputs applied to a potato culture in the Andean region, AM inoculum were combined with a different kind of management. An attempt was made to contrast in the field both kinds of management: the traditional with very high inputs, and a more conservative using AMF inoculum without any management.

In these soils, very aggressive populations of nematodes and mites are present. For this reason, the management applied currently by the farmers include the application of nematicides (20 kg/ha) at the moment of sowing and at least 10–12 applications of insecticides and fungicides 1 month after sowing. The first six doses are applied weekly, and then every 2 weeks to the end of the crop cycle. Additionally, 250 kg/ha of lime and 3–4 l/ha of herbicide are applied just when the plants get established and again 1 month after sowing. In addition, chicken manure (4 ton/ha) is introduced at sowing. Chemical fertilizer in the form of NPK (50 g/plant) and foliar fertilizer (N) and microelements (300–500 g/200 l) are also applied 1.5–2.5 months after sowing. Finally, after flowering, a chemical fertilizer rich in potassium is added.

In this case (Table 4), the highest yield of potatoes was obtained in the stand with very high inputs and inoculated with *Entrophospora colombiana* (15.6 ton/ha) or with *Gigaspora ramisporophora* (15.5 ton/ha). However, the yield of both treatments

Table 4 Effect of two management systems and inoculation with AMF on potato production in a potato culture in an Andean farm

	Potato yield (ton ha^{-1})	% MA	% Arbuscules
Without inputs			
Noninoculated	8.84	23.6	0
E. colombiana	7.87	22.6	0.6
Gi. ramisporophora	2.35	26.5	0
G. manihotis	5.60	8.9	0
S. fulgida	2.33	22.4	0
A. lacunosa	3.30	6.4	0
Very high inputs			
Non-inoculated	12.65	27.2	0
E. colombiana	15.59	18.6	0
Gi. ramisporophora	15.47	9.0	0
G. manihotis	12.16	10.8	0
S. fulgida	12.26	17.8	0.2
A. lacunosa	8.45	0.2	0

For statistical analysis, see text

was similar (statistically) to that obtained in uninoculated treatment (Table 4). The interpretation of these results could be that in the soil the indigenous AMF community is very efficient in promoting potato growth. However, the analysis of mycorrhizal colonization indicates a very low rate of AM-infection and the absence of arbuscule in all treatments performed (Table 4). In this case, it seems evident that the yield obtained was a consequence of the applied fertilizers and agrochemicals and not from AM inoculation. AMF colonization was scarce and probably AM did not function at all. Indeed, ANOVA results showed a nonsignificant effect of AMF inocula ($p = 0.07$), but a highly significant effect of management ($p = 0.00$). The interaction between the two factors was also not significant ($p = 0.27$).

Despite of the high amount of inputs applied, the yield obtained was low in comparison for example with the national mean rate for Venezuela (Linares and Gutiérrez 2002), which is about 18.7 ton/ha. So not only did these practices produce damage to the environment but they are in addition useless, as the yield obtained can be considered as marginal.

It is necessary to perform a great effort to change this situation despite of the well-nown fact that any changes in agronomic practices are difficult to impose on the growers (Vosatka and Dodd 2002), who usually are very conservative in their mentality, a fact which is understandable because farmers are usually those who cope with the higher risks and generally are not those who receive the highest benefits. As a consequence, the unlimited use of chemical fertilizers and agrochemicals is causing not only environmental problems (Carvalho et al. 1998), but also it is affecting human health (Dua et al. 1996).

2.4 Production at an Agronomic Scale

Plantain culture (*Musa* AAB) is a very important commodity in tropical and subtropical regions. Its global production is around 88 million tons/year. It constitutes the main food source for at least 400 million people (Sharrock and Frison 1998; FAO 2001). There are no published data up to now regarding the response of plantain to AMF inoculation in the field. However, there are several works regarding the AM dependency of another close-related crop, the banana (Declerck et al. 1995; Jaizme-Vega and Azcón 1995; Melo et al. 1997; de Oliveira and de Oliveira 2005).

The effect of AMF inoculation on plantain yield was evaluated in a commercial plantation (Table 5). In the Caribbean region, it is managed like a perennial crop. It is sown in 3 × 3 m design and fruits are harvested 10–12 months after sowing. This species has high nutritional requirements especially regarding K, but the soils used for its production – in this particular case, tropical soils with moderate to high nutrient content – normally can cover these requirements. However, farmers with access to economical resources apply fertilizers – even if they seem not very necessary – and fumigate against Sigatoka Negra disease (*Mycosphaerella fijensis*), a fungus which attacks plantain in tropical countries. Inoculation of plantain with *Scutellospora heterogama*, in the presence or not of fertilizers, produced a yield increase of 6 times

Table 5 Effect of AMF inoculation and fertilization on fruit yield in a plantation of plantain (*Musa* AAB) in the field

Treatments	Yield (kg plant^{-1})
Noninoculated	1.32 a
Mixed inoculum A**	0.00 a
Mixed inoculum B**	1.41 a
Glomus manihotis	3.00 ab
Scutellospora heterogama	7.94 bc
Fertilization *	9.23 cd
Mixed inoc. A** + Fertilization*	13.27 def
Mixed inoc. B** + Fertilization*	11.86 cde
G. manihotis + Fertilization*	14.73 ef
S. heterogama + Fertilization*	17.12 f

* 250 g/plant of NPK (200:30:400) every 3 months

** A, B are mixed AMF inocula produced starting from AMF communities associated with two different plantain plantations

Means followed by the same letter are not significantly different, $p < 0.05$

compared with the uninoculated treatment (indigenous AMF), in the absence of additional fertilization (Table 5). When the inoculation was accompanied with fertilization an increase of 1.8 times in fruit production was obtained when compared with the treatment fertilized-noninoculated (Table 5). In contrast, inoculation with *Glomus manihotis* or with two other mixed inocula isolated from plantain plantations do not produce a significant increase in yield in comparison with their respective controls. In summary, even in soils not low in P (available P in this soil is 16.5 ppm); it is possible to find an AMF which can contribute an increase in yield.

Another crop that can have industrial applications in tropics is cassava, which until recently was considered a marginal crop. However, areas used to sow cassava tend to be very acidic and nutrient-poor as was described previously. In an experiment performed in the east plains of Venezuela it was possible to substitute the half of the fertilizers normally applied by farmers in such low in nutrient soils (available P 1.7 ppm), by an inoculum of *Scutellospora fulgida*, to obtain the same tuber yield (Table 6). However, cassava in Nigeria has been cultivated with AMF in unsterilized soil and in the absence of fertilizers, even in nutrient-poor soils, with success (Salami and Osonubi 2003). The simultaneous mulching of cassava with 0.5 ton/ha of tree-legume prunings (*Gliciridia sepium* and *Senna siamea*), together with the AM inoculation in the field, increased the root yield of cassava from 10 ton/ha (with AMF alone) to 35 ton/ha (with AMF plus mulching), thus substituting fertilizers by mulching (Salami and Osonubi 2003). On the other hand, it has been shown that the responses of crop growth and yield to different organic amendments may be related to changes in populations of AMF (Muthukumar and Udaiyan 2002). In the humid tropics, where many farmers cannot afford the use of inorganic fertilizers due to very high cost or logistic problems of distribution, mycorrhiza inoculation plus mulching may provide an useful alternative which in addition does not compromise environmental integrity.

Table 6 Effect of AM inoculation and fertilization on tuber yield of cassava (*Manihot esculenta* var Paiguanera) in the field

Fertilization rate	AMF inoculum	Tuber yield (ton/ha)
None	Noninoculated	34.0 ab
None	*Glomus manihotis*	31.2 ab
None	*Acaulospora lacunosa*	39.2 ab
None	*Scutellospora fulgida*	30.7 a
Half	Noninoculated	48.7 ab
Half	*Glomus manihotis*	50.5 ab
Half	*Acaulospora lacunosa*	50.5 ab
Half	*Scutellospora fulgida*	54.5 ab
Complete*	Noninoculated	60.0 b
Complete	*Glomus manihotis*	52.7 ab
Complete	*Acaulospora lacunosa*	59.2 ab
Complete	*Scutellospora fulgida*	38.0 ab
Organic	Noninoculated	36.0 ab
Organic	*Glomus manihotis*	36.7 ab
Organic	*Acaulospora lacunosa*	39.7 ab
Organic	*Scutellospora fulgida*	41.2 ab

*Complete fertilization consisted of 330 kg/ha of NPK (12:29:12) plus 50 kg/ha $ZnSO_4$, 15 days after sowing, and 140 kg/ha KCl plus 133 kg/ha of urea, 45 days after sowing. Means followed by the same letters are not significantly different, $p < 0.05$

2.5 Slash and Burn Agriculture

The highly biodiversity forest of Amazon basin represent the world largest tropical wilderness area, and are home to hundreds of thousands of indigenous people, many of whom maintain a predominantly traditional lifestyles (Geist and Lambin 2002).

The slash and burn agriculture system was developed by inhabitants of tropical forest thousands of years ago. In the Amazon Basin it was, and still is, the most important form of subsistence, and most farming tribes use this method (Saldarriaga 1987). The traditional methods of agriculture practiced by the first inhabitants of this region were characterized by slashing and burning mature forest or secondary vegetation (Fig. 1); followed by continuous cultivation of diverse mixture of crops and then abandonment after 3–5 years, depending upon fertility of the soil and the care of the owners finally abandoning the site when productivity dropped (fallow). These agricultural systems are locally called *conuco* by indigenous as well as by local people. The main crops used by Piaroa communities in the Amazon Basin are cassava (*Manihot esculenta*), maize (*Zea mays*), pineapple (*Ananas* spp), *Dioscorea* sp. and other autochthonous fruits (Jordan 1987; Nicholaides et al. 1985).

There is high productivity during this first culture period, but in a few years leaching of nutrients and erosion caused by heavy rains on deforested areas produce a drastic reduction in yield. Uhl and Murphy (1981) recorded the reduction of

Fig. 1 Slash and burning of mature forest performed by indigenous people in Venezuelan Amazonas

productivity in a culture of cassava from 5.3 to 1.4 tons/ha/year, between the first and the third year of culture in *conucos* of San Carlos de Rio Negro, Amazon.

Many traditional indigenous land uses have historically been ecologically sustainable in the context of low population densities and isolation from external markets, but transformations of indigenous livelihoods include the adoption of unsustainable practices actually putting into question the long-term sustainability of indigenous forest management (Escalante and Arends 2001). The new practices are not adapted to the soil limitations of the fragile Amazonian ecosystems, endangering the stability of the environment in which the Piaroa communities are established (Royero et al. 1999). The increase in crop production in these areas is one of essential steps to meet the food demand of the growing human population. The progressive change from nomad to sedentary pattern of life is causing more demands on soil and plant resources, reducing fallow periods after *conuco* is abandoned. The utilization of fallow land was drastically shortened to less than 7 years, contributing to the "savannization" of the rain forest and to a drastic drop of productivity (López-Hernández et al. 1997).

AMF are native to all tropical soils and it can be assumed that the actual vegetation and crop production under tropical nutrient deficiency is largely dependent on the indigenous AMF fungal population. However, the distribution of AMF in soil is not homogeneous, and there are vegetation and crop production systems where the soil inoculum potential is too low for optimum plant biomass production. In such cases, use of commercial inoculum may be the best way to increase crop productivity (Sierverding 1991).

After burning the forest, the number of spores diminished drastically in comparison with *conuco* (Table 7). As in tropical America, the preferred crop that is sown in slash and burn agriculture is cassava (*Manihot esculenta*) which is a species highly dependent on AM (Sieverding 1991), the plant succession that follows the *conuco* stage results with enough AMF inoculum (Cáceres 1989). Generally, weeds and sedges invade the abandoned *conuco* and plant succession starts again. Indeed, the evaluation of AM colonization in different crop plants utilized by Piaroa communities in an established *conuco*, showed high values of colonization and increased of inoculum potential, compared with forest and fallow after 6 years abandoned (Tables 8 and 9) (Cáceres and Kalinhoff 2003).

AM inoculum recently produced in Venezuela (Cuenca et al. 2007) was applied by Piaroa native communities in Venezuelan Amazon (Cáceres and Kalinhoff 2003). As these communities do not have access to fertilizers nor agrochemical substances, AM inoculum was applied alone in the planting hole (100 g per plant), and cassava was planted in the traditional manner (Fig. 1). The results indicated an increase in fresh weight of roots and height of *M. esculenta* inoculated in comparison with noninoculated plants (indigenous AMF). *Glomus manihotis* and *Scutellospora fulgida*, produced a significant increase in cassava root weight and height (Table 10) and *Acaulospora lacunosa* was the less effective fungus. Sierverding (1991) proposed that the response of cassava to field inoculation is not significant when more than 900 infective propagules/100 g dry soil were present, thus in this case field inoculation can be an adequate practice to increase productivity in *conuco* because the infective propagules were low (see Table 9).

Table 7 Number of AMF spores/100 g soil present in soil forest 15 days after burning for the establishment of *conuco* and spore number present when *conuco* is finally established

Management stage	Number of AMF spores/100 g
Forest, 15 days after burning	0.7 ± 0.4 a
Conuco	389.5 ± 48.5 b

Different letters indicate significant differences ($p > 0.05$)
Source: Cáceres (1989)

Table 8 Presence of AM colonization in plants growing in a farm (conuco) after slash and burning in Amazon

Species	% AM colonization
Manihot esculenta	59
Theobroma grandiflorum	47
Zea mays	76
Dioscorea alata	85
Pouteria caimito	61
Ananas comosus	50

Source: Cáceres and Kalinhoff (2003)

Table 9 Most probable number (MPN) of AMF propagules at different sites in Amazon

Management stage	MPN/100 g soil (95% confidence interval)
Forest	69 (32–146)
Conuco	603 (281–1,288)
Fallow (6 years after abandonment)	120 (56–257)

Source: Cáceres and Kalinhoff (2003)

Table 10 Fresh weight of roots and height of *Manihot esculenta* inoculated with three different AMF in comparison with indigenous AMF inoculum

Treatments	Fresh weight of tuber (kg/plant)	Mean shoot height (m/plant)
No inoculated	0.77 ± 0.15 a	2.34 ± 0.06 a
Glomus manihotis	1.55 ± 0.09 c	3.09 ± 0.12 b
Acaulospora lacunosa	0.96 ± 0.12 b	2.96 ± 0.11 a
Scutellospora fulgida	1.65 ± 0.16 c	3.24 ± 0.08 b

Different letters indicated significant differences ($p < 0.05$)
Source: Cáceres, Kalinhoff and Romero, unpublished results

It is obvious that the efficiency of indigenous AMF community was lower than the introduced one. However, it should be clear that effectiveness of indigenous AMF fungal population is related to multiple factors such as the soils nutritional status, the host plant, propagules density and the competition between them and other soil microorganisms (Azcón-Aguilar et al. 1986; Sieverding 1991).

Indigenous people abandon *conuco* when productivity drops, and this is a traditional management practice for restoration of soils that requires a long fallow period for sufficient regeneration of the native vegetation and establishment of tree species (Uhl 1987; Barrios et al. 2005; Toledo and Salick 2006). One alternative to traditional fallow is planting valuable perennial crops such as fruits trees among the early crops, so that there is a continuity of production. That could improve soil nutrient stock faster than plants in natural succession, so indigenous fallow management should include harvesting, weeding, planting and maintaining useful plants (perennial crops) that can represent production for family consumption. This is an appropriated management because it can generate additional products that bring immediate benefit to indigenous communities while improving soil fertility (Barrios et al. 2005).

3 Research and Priorities for the Future

Results presented here seem to leave no doubt about that many tropical areas are still following the principles of the so-called “green revolution” which is producing crop commodities utilizing the highest amount of chemical and agrochemical that

can be handled. Consequences of this behavior can be easily seen easily in the strong contamination present in rivers and lakes that in many cases show evident signs of eutrophication.

However, in other areas, there are another more ecologically suited alternatives which use organic and green manure, agro-forestry, phosphate rock, mycorrhizas, etc., to thrive with the nutritional deficit that characterizes the majority of tropical soils. But it is also clear that production in this type of soil cannot be based exclusively on the use of arbuscular mycorrhizal inoculum. Despite evidence that shows that AMF are able to utilize nonavailable sources of nutrients in artificial media (Koide and Kabir 2000; Cardoso et al. 2006), the magnitude of such effects in field conditions have not been measured, and doubts remain whether AM fungi can utilize P that is naturally fixed or organically bound in soil (Cardoso and Kuyper 2006). So, it is necessary to agree with the idea that, in soils with low levels of available nutrients, AMF inoculation should be accompanied by a small dose of fertilizer, enough to power the AMF functioning but not so high as to cause the inhibition of AMF formation and produce contamination.

Without doubt, a sustainable agriculture should be adopted seriously as a new paradigm for humanity. In consequence, the agriculture for the less developed countries should modernize and substitute the old practices from the green revolution with a new agriculture based in soil biology (Cardoso and Kuyper 2006). Evidence shown here indicate that is possible to obtain the same products sparing a great amount of chemicals, improving in this way the environmental quality.

However, while the consequences of the global movement of AMF inoculants are unknown (Schwartz et al. 2006) it is necessary to establish rules that carefully control the free entrance of AMF inoculum from other very different environments through every frontier, and to request the existence of strict quality controls for these materials. In addition, it is necessary to undertake strong efforts in education both for the public and the farmers about the problems and possible solutions pointed out here. If priorities for people from tropical countries include these items, the achievement of a sustainable agriculture even in the poor nutrient condition characteristics of acidic tropical soil will be a reality.

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References

Azcón-Aguilar C, Barea JM, Azcón R, Díaz-Rodríguez RM (1986) Assessment of field situation for the feasibility of vesicular–arbuscular mycorrhizal inoculation, using a forage legume as test plant. Agric Ecosyst Environ 15:241–252

Barrios E, Cobo J, Rao I, Thomas R, Amezquita E, Jiménez J, Rondon M (2005) Fallow management for soil fertility recovery tropical Andean agroecosystems in Colombia. Agric Ecosys Environ 110:29–42

Brady NC, Weil RR (2002) The nature and properties of soils, 13th edn. Prentice Hall, Upper Saddle River, N.J.

Cáceres A (1989) Las micorrizas vesículo arbusculares en un bosque húmedo tropical y su evolución luego de la perturbación (conuco) y la sucesión por 60 años en San Carlos de Río Negro, T.F. Amazonas. MSc Thesis. Instituto de Investigaciones Científicas, Venezuela

Cáceres A, Kalinhoff C (2003) Efecto de la perturbación producida por el establecimiento del conuco sobre las micorrizas arbusculares (MA). Technical Report. Fonacit ULA-UCV

Cardoso IM, Kuyper TW (2006) Mycorrhizas and tropical soil fertility. Agric Ecosys Env 116:72–84

Cardoso IM, Boddington C, Janssen BH, Oenema O, Kuyper TW (2003) Disribution of mycorrhizal fungal spores in soils under agroforestry and monocultural coffee systems in Brazil. Agrofor Syst 58:33–43

Cardoso IM, Boddington C, Janssen BH, Oenema O, Kuyper TW (2006) Differential access to phosphorus pools of an Oxisol by mycorrhizal and non mycorrhizal maize. Comm Soil Sci Plant Anal 37:1–15

Carvalho FP, Nhan DD, Zhong C, Tavares T, Klaine S (1998) Rastreo de plaguicidas en los trópicos: Resultados de un proyecto internacional de investigación. Boletín OIEA 40:24–30

Cuenca G, De Andrade Z, Escalante G (1998) Arbuscular mycorrhizae in the rehabilitation of tropical fragile degraded lands. Biol Fertil Soil 26:107–111

Cuenca, G, De Andrade Z, Meneses E (2001) The presence of aluminum in arbuscular mycorrhizas of *Clusia multiflora* exposed to increased acidity. Plant Soil 231:233–241

Cuenca G, De Andrade Z, Lovera M, Fajardo L Meneses E (2003) Mycorrhizal response of *Clusia pusilla* growing in two different soils in the field. Trees 17:200–206

Cuenca G, Cáceres A, Oirdobro G, Hasmy Z, Urdaneta C (2007) Las micorrizas arbusculares como alternative para el desarrollo de una agricultura sustentable en áreas tropicales. Interciencia 32:23–29

da Silveira, SV, de Souza PVD, Bender RJ, Koller OC (2002) Effect of arbuscular mycorrhizae on cv. Carmen avocado plants. Commun Soil Sci Plant Anal 33:1323–1333

de Oliveira AN, de Oliveira LA (2005) Colonização por fungos micorrízicos arbusculares e teores de nutrientes em cinco cultivares de bananeiras en um latossolo de Amazônia. R. Bras. Ci. Solo 29:481–488

Declerck S, Plenchette C, Strullu DG (1995) Mycorrhizal dependency of banana (*Musa acuminata* AAA group) cultivar. Plant Soil 176:183–187

Dua VK, pant CS, Sharma VP (1996) Determination of levels of HCH and DDT in soil, water and whole blood from bioenvironmental and insecticide-sprayed areas of malaria control. Ind J Malariol 33:7–15

Escalante E, Arends E (2001) Sistemas agroforestales para la rehabilitación de áreas intervenidas y degradadas en la Reserva Forestal Sopapo. Memorias del IV Congreso Venezolano de Ecología, Sección Ecología Forestal p 136 Mérida, Venezuela

FAO (2001) http://*www.fao.org*

FIDA (International foundation for agriculture development) (2003) La adopción de la agricultura orgánica por parte de los pequeños agricultures de América Latina y el Caribe. Technical report number 1337

Geist H, Lambin E (2002) Proximate causes and underlying driving forces of tropical deforestation. Bioscience 52:143–150

González EJ (2004) *Lemna* en el Lago de Maracaibo (Venezuela). Artículo publicado en la página web de la Red de Eutrofización de Lagos y Embalses (http://rele.fcien.edu.uy/publicaciones/)

Infante A, Infante O, Márquez M, Lewis WM, Weibezahn F (1979) Conditions leading to mass mortality of fish and zooplancton in Lake Valencia, Venezuela. Acta Cient Venez 30:67–73

Jaizme-Vega MC, Azcon R (1995) Responses of some tropical and subtropical cultures to endomycorrhizal fungi. Mycorrhiza 5:213–217

Jeffries P, Barea JM (2001) Arbuscular mycorhiza - a key component of sustainable plant-soil ecosystems In: Hock B (ed) The mycota IX fungal associations. Springer, Heildelberg, pp 95–113

Jordan CF (1987) Shifting cultivation: slash and burn agriculture near San Carlos de Río Negro, Venezuela. In: Jordan CF (ed) Amazonian rain forest: ecosystem disturbance and recovery. Ecological Studies. Springer, Heidelberg, pp 9–23

Knapp B, Ashby JA, Ravnborg HM (1996) Natural resources management research in practice: the CIAT Hillsides agroecosystems program. In Preuss, H-J.A. (ed.). Agricultural Research and Sustainable Management of Natural Resources. LIT Verlag. Munster-Hamburg. Germany, pp 161–172

Koide R, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolise organic phosphate. New Phytol 148:511–517

Linares Y, Gutiérrez A (2002) La competitividad de la producción de papa (*Solanum tuberosum*) en el Municipio Pueblo Llano, Estado Mérida, Venezuela. Agroalimentaria 15(12)

López-Hernández D, García–Gaudilla MP, Torres F, Chacón P and Paoletti MG (1997) Identification, characterization and preliminary evaluation of Venezuelan Amazonian production systems in Puerto Ayacucho savanna–forest ecotone. Interciencia 22:307–314

Melo AM, Maia IC, Morgado LB (1997) Fungos micorrízicas arbusculares em bananeiras cultivadas no Vale do Submédio São Francisco. Acta Bot Bras 11:115–121

Muthukumar T, Udaiyan K (2002) Growth and yield of cowpea as influenced by changes in arbuscular mycorrhiza in response to organic manuring. J Agron Crop Sci 188:123–132

Nicholaides III JJ, Bandy DE, Sánchez PA Benites JR, Villachica J H, Coutu AJ, Valverde CS (1985) Agricultural alternatives for the Amazon Basin. BioScience 35:279–285

Osorio NW, Alzate JM, Ramírez GA (2002) Coffee seedling growth as affected by mycorrhizal inoculation and organic amendment. Commun Soil Sci Plant Anal 33:1425–1434

Ramírez R (2006) Eficiencia del uso del fósforo de la roca fosfórica por cultivares de maíz. Interciencia 31:45–49

Royero R., Narbaiza I, Narbaiza A, Contreras J, Vele G (1999) Base de datos para la información agroalimentaria, de la tecnología ancestral y medicina nativa para el desarrollo sustentable en diversas etnias de Amazonas. Mem Inst Biol Exp 2:85–88

Salami AO, Osonubi O (2003) Influence of mycorrhizal inoculation and different pruning regimes on fresh root yield of alley and sole cropped cassava (*Manihot esculenta* Crantz)l in Nigeria. Arch Agron Soil Sci 49:317–323

Saldarriaga J G (1987) Recovery following shifting cultivation: a century of succession in the upper Río Negro In: Jordan CF (ed) Amazonian rain forest: ecosystem disturbance and recovery. Ecological Studies. Springer, Heidelberg, pp 24–33

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Sharrock S, Frison E (1998) Musa production around the world-trends, varieties and regional importance. In: Networking banana and plantain: Annual report 1998. INIBAP, Montpellier, France

Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. GTZ, Eschborn, Germany

Smith S, Read D (1997) Mycorrhizal symbiosis. Academic, San Diego, Calif.

Toledo M, Salick J (2006) Secondary succession and indigenous management in semideciuous forest fallow of the Amazon basin. Biotropica 38:162–170

Torres D, Capote T (2004) Agroquímicos un problema ambiental global: uso del análisis químico como herramienta para el monitoreo ambiental. Ecosistemas (digital journal) *http://www.revistaecosistemas.net/*

Uhl C (1987) Factors controlling succession following slash-burn agriculture in Amazonia. J Ecol 75:377–407

Uhl C, Murphy P (1981) A comparison of productivities and energy values between slash and burn agriculture and secondary succession in the upper Río Negro of the Amazon Basin. AgroEcosyst 7:63–83

van Noordwijk M, Cadish G (2002) Access and excess problems in plant nutrition. Pl Soil 247:25–40

Vosatka M, Dodd JC (2002) Ecological considerations for successful application of arbuscular mycorrhizal fungi inoculum. In Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. From genes to bioproducts. Birkhäuser, Basel, pp 235–247

The International Market Development for Mycorrhizal Technology

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1 Introduction

During the last few years there has been remarkable boom in enterprises producing mycorrhizal fungi inocula and related services for the retail sector, commercial plantations, horticulture and, more recently, the developing agricultural market. Customers range from the general public and commercial growers to governments, each requiring a different market focus and varying degrees of after sales support. There is a number of reasons for this growth in the mycorrhizal industry. In particular, the increasing body of scientific evidence on the positive effects of mycorrhizal fungi on plant health, growth and yield has driven a greater awareness by end users of mycorrhizal technology. In addition, there is a realisation of costs/benefits of mycorrhizal technology, through its use in large-scale projects. Especially, there has been recognition in the market that microbial products offer a sustainable approach to plant production, one that is both environmentally friendly and economically attractive in commercial cultivation.

Possibly a more interesting feature of the commercial evolution of the technology is the high number of companies that have 'spun out' of research institutes or have a mycorrhizal scientist in their founding teams or as consultants. This fact illustrates that in this field of research the distance between applied research and commercial product is very short. Indeed, many researchers have viewed commercial activity as a more effective way to undertake large-scale applied research.

The development of an industry producing arbuscular mycorrhizal fungi (AMF) inocula embraces not only the development of the necessary biotechnological know-how, but also the ability to respond to specific legal, ethical, educational and commercial requirements (Vosatka et al. 1999; Gianinazzi and Vosatka 2004).

The credibility of all commercial products in this field will be enhanced by the research endeavor across the globe. Significant developments in the mycorrhizal

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fungi research effort that will support the market's understanding and interest in mycorrhizal fungi products will include the development of molecular probes for monitoring AMF inocula in the field (Graham and Miller 2005) and increasing knowledge on both the ecophysiology of AMF in disturbed ecosystems and on the interactions of AMF with other rhizosphere microbes (Hawkes 2003; Johansson et al. 2004; Selosse et al. 2004). However, the main task for both inoculum producers and researchers is to raise awareness in the public about the effectiveness and ecological significance of mycorrhizal technology in commercial and domestic planting. The very fact that an industry exists suggests that the body of evidence concerning the positive effects of mycorrhizal fungi is sufficient to develop the market. It is important that researchers and producers now build on this success to promote applied work to show the effects of the technology in different locations, environmental conditions and under different stresses.

2 Commercialization

In nature, mycorrhizal fungi play a key role in the sustainability of plant life. As we can now produce good quality mycorrhizal inoculum it is clear that we can now apply this critical biological component to commercial horticulture and agriculture. Even if we take an extremely conservative view of the application of this technology, the market potential is both global and huge. The task facing the industry is how to develop business models to exploit the market sectors and how to evolve from domestic to international and potential multinational operations.

2.1 Market Developments

To date, there are about 12 domestic producers (companies producing inoculum for direct retail) in the EU, including: PlantWorks in UK, Symbio-m in the Czech Republic, INOQ in Germany, MYCOSYM in Switzerland, SYTEN in Spain, Biorize in France, etc., and more than 20 others worldwide (the majority of the others residing in the USA).

The most developed retail market for mycorrhizal fungal products is North America – Première Tech leading the retail market in Canada and a more diverse USA market. Significant commercial operations in this market have attracted venture capital funding, acquisitions by larger companies (Roots Inc. acquired by Danish Novozymes A/S) and more recently public flotation (PlantHealthCare Inc, IPO 2004 on the Alternative Investment Market of the London Stock Exchange). Of the current USA-based companies, these two producers have shown proactive marketing activity outside their own country – they have become international operations.

The general market in the European Union (EU) for mycorrhizal fungi products remains 'immature' with many producers enjoying a monopoly in their home country. Competition is limited to minor penetration by other suppliers into domestic

markets. Early signs of the development of international distribution can be seen with producers developing distribution channels into other countries including the USA, but to date the original pioneers still maintain dominant positions in their local and regional markets.

When a market is 'immature', the pioneer companies in that market can develop a customer base by direct interactions and networking. As competition arrives, the marketing process becomes more streamlined with a professional focus on product differentiation through the development of products with different functions, packaging and brand. Ultimately though, in any commodity market we see a convergence of products in relation to function and price. At this juncture of market evolution, the mode of competition will move from differentiation to cost. When the market has developed to this phase, the key focus will be on the cost of production as, ultimately, those who can produce the most cheaply can maintain profits while reducing selling prices to win customers. This logic has far-reaching consequences in terms of the 'bet' as to which of the current producers will become dominant when this market ultimately matures.

If pure logic dictates our futures (and it rarely does) we can take an objective view as to how the dynamics of political, economic, social, and technological forces will serve to shape this industry in the coming years, and which companies are the best placed to take advantage of this evolution.

2.2 Political Forces

The current market for mycorrhizal fungi products has, to date, remained relatively unrestricted by political forces. In Europe, no directive specifically controls the manufacture, use or movement of these products. There is no unifying regulation in Europe covering mycorrhizal fungi products, where they currently are in relation to pure fertilizers. Regulations pertaining to the fertilizer industry cover the health and safety of employees and the general public, conditions for the safe storage and transportation of manufactured fertilizer material and intermediates, limits on emissions to the atmosphere and water (fresh and marine), limits on noise levels, and the treatment and disposal of waste products resulting from the production of fertilizer or fertilizer intermediates.

Regulation for mycorrhizal fungi products is limited to product registration in some countries, e.g., France, Czech Republic, Hungary and others, where samples of the product are required for testing in relation to composition and function (efficacy). Registration can be expensive and acts as a barrier to entry to competition. Although registration serves as a level of protection for domestic producers it also clearly restricts competition and the associated drive on product prices and choice. In the developing stages of the market this may serve to limit the development of the market in countries where registration is required, giving rise to the conflicting market philosophies of the protectionists and free marketeers.

It is surprising in some respects that the market for microbial products has managed to stay unregulated in a political environment where the majority of

agricultural and environmental activities have attracted so much attention. It may be prudent therefore to assume that the situation, where partial lack of regulation exists, may presently come to an end. Regulation, whether it will be driven from the US or EU, or imposed by some worldwide organization, will affect all international producers. The need to lobby the regulators to ensure that regulations function to protect the market from inferior products but do not inhibit trade has not gone unnoticed in Europe. Driven by these concerns, the existing European producers have established a "guild" association of producers to promote quality standards in the industry. The strategic value of the association 'The Federation of European Mycorrhizal Fungi inoculum Producers' (FEMFip, www.femfip.com) is manyfold: it promotes quality across all companies in Europe; it promotes interaction between European research groups and producers, and it acts as a common voice to the European Commission. The key function is though the promotion of quality; in this regard, the association requires very tangible tests on products offered for sale by its members, employing an agreed 'most probable number' (MPN; Gianinazzi-Pearson et al. 1985) protocol on random samples.

FEMFip exists as the proactive 'trade association', but others have been established, namely the International Mycorrhizal Manufacturers Association (IMMA; http://www.hortsorb.com/News_Releases.asp) in 2003, mainly comprising North American producers of mycorrhizal inoculants. The stated mission of the IMMA is to collectively oversee the maintenance of high standards of quality for mycorrhizal products worldwide to ensure the future viability of the industry. The recently-established (2006) International Mycorrhizal Society (IMS; http://www.mycorrhizas.org/) aims to incorporate producers internationally worldwide to maintain the good name of mycorrhiza and mycorrhizal products.

Outside the EU, the dominant regulations that are of concern are those restricting the import and export of goods, and these by definition will control to scope of the larger producers evolving from national to international companies. International distribution of fungal inocula containing other microorganisms is an issue consisting of two matters: firstly, it involves distribution of potentially nonindigenous organisms and, secondly, the associated risk of dispersing unwanted harmful organisms. Import/export issues vary significantly across the globe, including: the requirement to obtain a phytosanitary certificate, proof of product origin and composition, translation of registration documentation (where they exists), and use of approved importers to full chemical analysis. One potential future virtue of mycorrhizal industry regulation may be to standardize global import/export requirements.

An interesting dynamic here is that those countries whose import restrictions (or associated cost for approvals) are too great, yet their markets are of significant value, will promote a change in the trading models used by producers. If international trade is not possible, i.e., the export of goods from one country to another, then multinational operations will have to ensue, such that companies will have to change from an export model to a model where they invest in new 'in country' manufacturing facilities. In the long-term, it could be argued that this will see the technology to develop globally more quickly, although this is predicated on the ability of the industry to invest in these models and may, in turn, rely on the financial institutions,

i.e., the World Bank, recognizing the global value of the technology to finance this method of expansion.

There is rarely just a single business model in any given market and the same is true for multinational expansion. For example, the company PlantWorks Ltd. (http://www.plantworksuk.co.uk/) has pioneered the establishment of incountry manufacture site licenses to exploit this technology, which significantly involves a target country on an economic level. The 'plantation model' jumps the import regulatory barriers by virtue of licensing stringent manufacturing protocols that employ a local substrate, labor and adapted local fungal isolates. This model has found political favor as it implicitly involves a high degree of 'controlled' technology transfer and involves local labor and raw materials resources. This model is currently employed globally in numerous cases for plantation of high value crops and government subsidized revegetation projects. However, it has a potential to evolve under joint venture agreements to retail surplus supplies to promote domestic market development and, by definition, to disseminate the benefits of the technology to a new audience. A cautionary word of warning about the adoption of this model, per chance it is perceived as a cheap expansion activity. Licensing requires significance management in relation to remote production, quality assurance, international culture, etc. Based on the experience of the Plantworks Company, it takes an average 2 years to negotiate and implement a multinational site license, and it requires that significant insurance liabilities be carried by the licensor. Payback is not instant, it is in fact a mid-term model for exploitation, but one which, when proven in a sector, can be replicated.

The link between academic researchers and commercial companies in the mycorrhizal field is thankfully very strong. In the EU, this is driven by the European Commission-funded COST actions that seek to bring together international applied research on a regular basis. As the forum is open to commercial companies, there is strong market focus to discussions that influence the direction of research programs for the betterment of the global market.

2.3 Economic Forces

Economic forces influence the market both in terms of the effect on cost of supply (and by association the retail price) and the purchasing capacity of the end market. In terms of manufacture, most companies employing the existing dominant manufacturing methods for AMF have key costs related to labor, substrates, and power costs.

Some existing European producers that have traditionally enjoyed low labor costs have seen an increase in fixed costs as wages have been forced into line with other nations as EU membership grows. The ratio of output volume of inocula to labor costs can be driven down by the use of mechanization or upscaling of production (economies of scale) and this will be a focus for many European-based producers in the coming years. For those producers in third world economies, labor costs will remain low for the foreseeable future.

An important proportional cost is that relating to power; most producers operate in glasshouses with dual-cycle production with artificial light and heating being employed to extend the cultivation season. Producers positioned in warm and more southern locations have an advantage as they can usually operate a third 'free' production cycle without costs for additional heating and light supplement. It could be argued therefore that those existing producers in, for example, Spain have a natural cost advantage in servicing their domestic markets. This cost advantage may extend out their domestic market and allow competitive pricing in other bordering markets, but the advantage will inevitably be balanced by the higher related transport costs compared to a home-based industry in a target country.

The majority of current inoculum producers use well-controlled 'closed' production facilities. The cost associated with the production, together with the profit margin required, defines the basic end price on products on the market. Due to the time and cost of production, the majority of producers target the high value markets where they can achieve a satisfactory margin. At the top end of this spectrum is the hobby garden market, where packaging and brands achieve premium pricing. Down from this sector are the municipal landscaping, horticultural and nursery markets. Very few northern hemisphere producers have the cost base to attempt to service agricultural markets at an attract unit price, due to the volume of inoculum required in most applications.

In the USA, some producers of high value crops, e.g., Advacos, have set up controlled AMF production areas, managed by trained mycologists, to ensure high quality and large volumes of inocula are available.

Agricultural markets are though being addressed in the southern hemisphere where labor rates are low and beneficial growing conditions exist. It is worthy of note that producers in India and Columbia produce more inocula than all northern hemisphere producers put together (estimated well over 1,000 tonnes annually in these two countries). A significant cost saving achieved by these producers is due to the open field production system employed that does not require high capital or maintenance expenditure. The issue with this approach, as with all mass production techniques, relates to quality and quality assurance management. Such widespread use of mycorrhizal fungi has begun to enter the planting culture in these countries and, when quality can be assured, will act as excellent promotional evidence for leverage by other producers globally.

3 Cultural and Social Aspects

Our review of the current global market and our conjecture on how market forces, that may effect it, may or may not be valid. What is for sure is that the shaping of the market will take some time, possibly 5–10 years from now. In the meantime, before competition develops to the point where some companies survive and some do not, there is a significant amount of market development to be undertaken that will be best achieved collectively by the current producers.

The biggest strategic challenge in the industry is one of changing a market culture from that of employing chemical intervention during planting to one based on

the use of a sustainable and ultimately more cost advantageous microbial approach. Although challenging, "cultural change" can be managed (Kotter 1996) through the accomplishment of a series of coherent milestones. Although not relevant for this review, the movement in market culture is a key activity for those marketing products into domestic and international markets of mycorrhizal biotechnology. The needs to promote the need to change, i.e., to develop a coalition of stakeholders interested in change and to establish short-term 'high impact' successes, are some of the key elements of this activity. Although theoretically possible, changing a global culture will be difficult; attempting this on a national level, although not easy, is achievable and will require local producers to engage with the local research community, media and end users. It is clear, therefore, that professional marketing of microbial products is not just a case of setting up a shop with good products for sale, but that it involves an understanding of how the market supply chain operates and when, where and how a 'substitute purchase' can be made from chemicals to mycorrhizal fungi.

One factor that tends to inhibit the broader use of mycorrhizal fungi is the common perception, promoted by many researchers and authors, that the most relevant use of AMF relates to revegetation and reclamation of disturbed soils where a plant encounters numerous stresses not only directly after transplanting but also in the entire course of cultivation. The lack of a mycorrhizal relationship is being recognised to be one of the major causes of poor plant establishment and growth in a variety of agricultural, urban and industrial landscapes. Although not untrue this view tends to dominant the market's consciousness.

In fact, if you seek a reduction in fertilizer, pesticide and fungicide additions then the application of AMF has the ability to be employed globally in markets from agriculture and domestic gardening to great functional effect. In addition, it is the only treatment available that has a potential to support the treated plant for its entire life span if no detrimental stressful conditions interfere. This is a broader message and one that the stakeholders in the market need to promote universally if the general planting culture is to change.

4 Technologies

The profitability of an industry can be maximized through premium pricing, where a meaningful differentiation can be found, and through a sustained focus on reducing manufacturing costs. In the global market place for AMF, the attainment of the latter task will not only maximize profits in the already developed markets but will open up new market sectors in agriculture.

Most current companies operating in the market compete with very similar manufacturing technologies for AMF, using conventional open pot hydroponic cultures employing various types of porous substrates enbaling development of mycelium in porous cavities from zeolite, expanded clay, attapulgite, vermiculite, etc. Although other techniques have been suggested, including aeroponic systems, the only other

currently employed comercial system is based on in vitro aseptic cultivation (the methodology developed and used by the Première Tech company).

Though in vitro aseptic cultivation production can be apparently preferred over the 'common' open pot hydroponic manufacturing methods (OPMM) stating its microbial purity, there is an important fact counterbalancing both production methodoligies, which is that OPMM must be successfully controlled regarding its inoculum effectivity and that quality systems are put in place to ensure product quality. The capital costs of the OPMM manufacturing system are modest, and the maintenance of the production cycle can be undertaken by a small number of trained operatives. The attractiveness of this system is also that the end product can be made from locally available substrates (clays, etc.) and that the processing equipment is not complicated to use. This has an advantage for startup ventures but, more importantly, these 'low tech' production methods are suitable for deployment in third world countries where labor rates are low, substrates are abundant and where there is a clear demand for AMF, for application in either subsistence or commercial farming.

Première Tech in Canada has developed industrially-viable innovative axenic production technology for certain strains of AMF. In vitro production of inocula is based on findings of several scientific laboratories that have demonstrated the capability of culturing AMF on transformed roots in vitro. The advantage of this approach is that the time of manufacturing cycle is significantly reduced as well as the size of manufacturing facilities, and there is less risk of contamination by unwanted microorganisms. Although increasing numbers of researchers are looking at this system, the strains available employing this system are not as comprehensive as those produced using more traditional methods OPMM at this time.

The in vitro manufacturing technology clearly has significant potential. Although the initial capital costs are likely to exceed those of more established methods of production, the unit costs of manufacture will probably be lower, with the additional merit that quality control can be managed precisely. Looking at these production technologies objectively, then both can produce high quality inoculum, both have relatively modest costs and both (ultimately) can produce a multispecies range of products. It may thus be contended that they are not significantly differentiated to allow one to dominate the market. Therefore, due to the market size, it could be suggested that both technologies will compete 'sustainably' with no one technology becoming dominant.

5 Selling Points and Marketing Strategies for Mycorrhizal Technology

To create a market it requires that products in the market address customer demands. By reviewing the findings from the global research activity that underpins mycorrhiza technology we can define the unique selling points (USPs) of AMF that may be 'packaged' to service a variety of global market demands:

1. Reduced mortality of treated plants, as the 'secondary fungal root system' provided by the mycorrhizal fungi ensures water and nutrients are transported to the plant in the critical early establishment phase of growth. This leads to fewer losses of plants, in particular under more difficult field conditions.
2. Reduced need for aftercare. The mycorrhizal fungi enable a treated plant to compete better against early colonizing weeds. Many early colonizer weeds are weak or nonmycorrhizal plants, and the beneficial mycorrhizal fungi can actually suppress their growth and restrict their nutrient uptake by favoring the treated mycorrhizal plants (Püschel et al. 2008). The extraradical mycorrhizal mycelium explore a greater volume of soil than the plants with only their own roots treated to make best use of available soil moisture thereby conferring a level of drought tolerance.
3. Increased stability of soils, anti-erosion action. Mycorrhizal fungi are the basis of a healthy soil community and stabilize soils both physically and biologically. AM fungi secrete a protein, glomalin, which is able to glue and aggregate soil particles enhancing nutrient availability while acting as an antierosion agent (Rillig 2004). The ability of mycorrhizal fungi to aggregate soil particles is a complementary key factor in points 4 and 5 below.
4. Increased plant nutrient uptake especially phosphates and trace elements. Due to the fact that a greater volume of soil is explored by mycorrhizal plants, which enables nutrients to be found that would normally be unavailable to the plants' own roots. The fine mycorrhizal mycelium is also able to absorb trace elements from soils that the plants' own coarser roots would not be able to access.
5. Increased tolerance to soil-borne pathogens (Dassi et al. 1998; Brimner and Boland 2003; Whipps 2004; Dalpe 2005). Plant roots have natural weaknesses (disease infection sites) that pathogens use to gain entry to the plant. Mycorrhizal fungi can also use these sites to colonize a plant root and effectively block them reducing a pathogen's ability to infect plants.
6. Increased tolerance of plants to grow on polluted or contaminated sites. The AM fungi are able to reduce toxic elements from being taken up by the plant by immobilizing them inside their own structures (Tonin et al. 2001; Gonzalez-Guerrero et al. 2005).

In following sections we will deal with the impact of different projects aimed at the use of mycorrhizal biotechnologies in different market sectors. It is important to understand the 'drivers' in any market sectors. While in the commercial sector the focus is usually in costs/benefits, for government funded projects, although price is never far from the top of the list, a higher priority is placed on biodiversity and sustainability. Treatment with mycorrhizal products encourages natural plant diversity through the interlinking of the fungal roots sharing nutrient resources amongst the plant community (van der Heijden et al. 2003).

Classic market theory defines a new market sector when one or more of the parameters of product price, product function, promotional strategy or method of distribution (place) change significantly (the four P's of the marketing mix). This holds true for the market sectors for mycorrhizal fungi that have been developed

to-date. In the following section, we explore these market sectors of mycorrhizal biotechnology world market that have been developed, or those which are developing, and review the research that underpins them.

5.1 Hobby Garden Sector

Many of the commercial activities of companies formed to exploit this technology have focused on the hobby garden market. Products, mainly comprising AMF and ectomycorrhizal fungi spores, have been formulated and packaged for retail. Although the distribution margins (the profit requirement of the retailer) are high, and in some European markets the retailer requires 100% profit on sales plus two times the Value Added Tax, the wholesale price per unit of inoculum is still attractive making the general sector attractive. Direct sales through mail or e-mail orders achieve higher margins as there are lower losses to third parties; mail order companies require margins in the region of 40–50% with direct Internet sales only suffering the e-commerce charges usually around 2% of sales value. The key marketing message is built around the use of mycorrhizal fungi as good (professional) planting practice for general garden plants.

The conflict in this sector is often the positioning of the products in the garden centre. Although organic and sustainable they are most commonly retailed in the fertilizer sector. This gives mixed messages to the gardener, as the true role of the product is to add a "secondary root system", which is able to support the host plant for its entire lifetime and not to offer a finite nutrient resource like a normal fertilizer. As there is a forced link between AMF and fertilizer, it is therefore feasible to value the end market for these products in this sector. This evaluation could be based on a percentage of the current fertilizer market, taking the view that mycorrhizal fungi products are in effect a substitute purchase for chemical fertilizer.

The appropriate strategy for marketing and application of mycorrhizal fungi requires an element of education and training. Companies should be committed to offering training and information to the market in the belief that, with all the facts available, end users will choose its products over those of its competitors. Mycorrhizal products should be designed to ensure that suitably treated plants do become mycorrhizal and that they are not being of biofertilizer nature with small or no amounts of mycorrhizal fungi added as a marketing gimmick.

5.2 Horticulture

Horticulture, formally the "science or art of cultivating fruits, vegetables, flowers, or ornamental plants" remains a significant market for mycorrhizal fungi products. This is a very attractive sector for all inoculum producers as it can also be formally marketed. It benefits from having clients with very large-scale operations, e.g., citrus plantations that when convinced of the benefit of inoculation can deliver significant repeat business.

Research and commercial trial work has quantified the costs/benefits in, for example, the application of mycorrhiza in flower production, where yield increases of over 15% have been reported on an annual basis. Further evidence from producers in demonstration trials has shown bulb flower yield increases in field production up to 50%.

Additional beneficial effects of an application of mycorrhizal technology in the horticulture sector are to ameliorate effects of soil pollutants. Recent studies have illustrated the capability of some AM fungi isolates to reduce translocation of heavy metals and possibly of other pollutants into edible aboveground parts of food crop, e.g., vegetables and tobacco (Tonin et al. 2001; Hutchinson et al. 2004; Janouskova et al. 2005). A unique sector of the horticulture market is the essential oil market where mycorrhizal fungi application can have tangible benefits. Although considerable amounts of commercial inocula are used in this pharmaceutical segment, concise scientific data on the interaction of AMF and essential oils production are still very scarce.

A very promising area of horticulture is the introduction of AM fungi in micropropagation where there is lack of beneficial microbes in in vitro cultivation, and consequently after post-vitro transplantation, and plants significantly benefit from introduction of mycorrhiza (Vestberg et al. 2002; Taylor and Harrier 2003).

5.3 Forestry and Landscaping

There are some newly emerging specific markets for AMF as well as ectomycorrhizal fungi for application on potentially large stands of fast growing trees (e.g., willow, poplar and alder) being planted in Europe as biomass plantations. A similar market sector comprises extensive plantations of fast-growing trees for timber in the tropics grown on clear-cut sites left after rainforest 'cropping'. Timber production in many tropical regions, for example, Latin America and Africa, is increasing significantly, and there is a growing market for endomycorrhizal inocula used in tree plantations of Acacia, species of *Podocarpaceae* and many others.

Another important sector for the implementation of mycorrhizal technology is in general landscaping where soils are usually subfertile, compacted or even polluted. Application in this sector is particularly advantageous on sites where aftercare, i.e., fertilization or irrigation, is difficult, such as when planting is undertaken on steep slopes or highway verges. In the majority of landscaping environments, soils are disturbed and contain only low levels of or no native mycorrhizal fungi. In such situations, these fungi may take years to naturally reestablish, and therefore the merit of inoculation at planting out is to establish an instant functional fungal root system that will combat transplantation shock.

Significantly, the sector of landscaping comprises corporate, government agencies (e.g. highways agencies) and municipality council customers, some with biodiversity policies that seek where possible to promote native planting and best reclamation practices to establish seminatural ecosystems. By employing native adaptive species of AMF in these sectors, producers can turn this 'restriction' into a positive marketing message.

The landscaping sector is a fragmented sector; a landscape architect working on behalf of a client usually specifies the use of mycorrhizal fungi, and therefore marketing effort is rarely focused on the client but rather down the supply chain. The market depends on planting projects becoming available, and although contracts can be significant in this sector, they are usually season-dependent and can be long-term (sometimes the supply contract can be spread over more planting seasons).

5.4 Re-vegetation and Remediation

Mycorrhizal fungi, when introduced into stressed and disturbed environments, provide a more natural and sustainable approach to plant establishment and growth, conferring the benefits of plant fitness, vigor and increased yield under adverse environmental conditions (e.g., examples of cultivation in volcanic ash fields in Iceland; Enkhtuya et al. 2003; Greipsson and El-Mayas 1999). The introduction of mycorrhizal fungi is feasible on these sites where native fungi are not present or denuded, such as volcanic ash, mine spoils, waste deposits or polluted sites (Oliveira et al. 2005), or where inappropriate soil and vegetation management has been employed (Harrier and Watson 2003; Plenchette et al. 2005; Gosling et al. 2006).

The market for phytoremediation has significant potential for AMF producers when it develops by virtue of mycorrhiza-promoting plant growth in harsh environments comprising contaminated or desertified soils (Vosatka 2001). Despite the fact that the direct role of AMF in the immobilization of pollutants, such as heavy metals, PAH or PCB compounds, including their phytoextraction (Christie et al. 2004; Zhang et al. 2005), has not been fully explored (Adriano et al. 2004), indirect positive effects of the AMF on plant fitness hold great potentials for mycorrhizal fungi use.

5.5 Agriculture

Developing the agricultural sector is possibly the biggest challenge for the existing inoculum producers both in terms of potential revenues and in global benefits. It is envisaged that application of mycorrhizal inoculum in this sector will not be by broadcast, due both to the requirement of placing the inoculum near to seed, or root zone, and to the wastage that would result. Instead, as has been demonstrated in commercial agricultural systems, fine-graded high-density inoculum would be applied through seed drilling.

There exists an interesting dynamic in this sector. It could be argued that the most developed agriculture market for mycorrhizal fungi is in the third world, where treatments are cost effective due to the cost advantages enjoyed by producers in the region. Possibly the highest value agricultural markets for such organic treatments that could replace chemical fertilisers are in western markets. These remain largely untapped due to the cost and margin requirements of western inoculum

producers. It could be argued that adopting less stringent open field production systems could be employed in western markets, but it is doubtful that the associated risks, in terms of product efficacy and the potential exposure to pathogens of this approach, would be acceptable in this sector.

This sector brings into focus an area of possible future collaboration between researchers and commercial producers in the quest for a consensus on 'efficacy limits' that if defined could help producers to develop this sector.

Let us take an example: If we use a 3-m seed drill with 14 drill arms with, say 2-cm exit holes, then the effective area we will treat in 1 ha is approximately 850 m^2. Let us then take an informed, but arbitrary, view of a good inoculum and propose that using an MPN rating of 1 with 1.3 million propagules/l is of value. Now let us set an 'efficacy limit' of say 1 propagule per sq cm, such that a seed put to ground by a seed drill would be within 0.5 mm of an active propagule capable of making the seedling mycorrhizal. Using this outline, we would need approximately 71 of inoculum for the treatment. In the majority of western markets this volume of inoculum would probably be expensive for all crops, but clearly due the cost and margin requirements in the third world this is not the case, hence the development of this market sector.

We could argue therefore that a new AMF production enhancement is required to develop the agriculture market in the developed western markets, one that can increase the propagule density in a standard production by 10-fold at the same unit cost. This may be one of the motives of some producers and research groups to develop new production techniques, and both the research community and financial institutions alike should clearly support this endeavor, as its commercial return as well as market impact could be significant.

Accepting that we have made some judgement on efficacy limits for this sector it is worthy of note that some western producers are promoting the use of 1 bag (600 g) of inoculum to treat 1.2 ha (3 acres), which almost brings to mind homeopathic dosage, with reported increases in crop yield.

6 Inoculum Productions and Quality Assurance

Basic formulations of AM fungal inocula currently available are very similar. The products usually contain all three propagules, e.g., hyphae, root fragment and fungal spores (components that can support the rapid establishment of a mycorrhizal fungi relationship under most conditions). Different formulated products are available on the market what creates the need for the establishment of standards for quality control.

The current industry as we know it has developed using different AMF strains and quite often ones not well characterized in terms of ecological requirements and stability. This situation has raised the need for this industry, in their own interest, to continually develop criteria that will satisfy minimum requirements of quality for the produced inoculum.

In this context, the following basic criteria of mycorrhizal product quality should be fulfilled by the companies developing this market: firstly, that the plants inoculated with the product must form a mycorrhiza and, secondly, that the AMF inoculum contained in the product must be free of agents, which could negatively affect normal plant growth and development. Another demand for a commercial product is that the shelf life of the inoculum should be sufficient to suit the end user markets. There is an absolute necessity that all AM fungi isolates used in a commercial product should have traceable provenance and preferably registration in some international, internet available database of fungal or microorganism genebank (it is not suggested that this information should be public domain but rather that a regulator could review the data if required).

The introduction of such criteria by the inocula producers could contribute to the definition of conditions for the registration products on national or international levels. Furthermore, in the product declaration, the following recommendations for quality standards may be considered: data on physical and chemical properties (pH, nutrient carriers) of the inoculum should be provided to end users, including the content and composition of additives. In addition, additives should be primarily aimed at supporting mycorrhizal development and thus should not just be a kind of general fertilizer.

The AMF propagule density is one of the major measures of inocula quality. The relevant number of propagules can be determined with various published techniques, e.g., MPN (most probable number) (Gianinazzi-Pearson et al. 1985) and IP (inoculum potential assay) (Liu and Luo 1994). MPN is more often applied, but results could vary according to the plant, substrate and the environmental conditions used (Feldmann and Idczak 1992). Therefore, there is a need for an independent testing service that can be used by producers to check that batches of inocula meet the baseline standards established and agreed by the relevant regulator or industrial trade association. Guaranteed efficacy of the products is usually very questionable, as the performance of fungi can differ significantly with soil and cultivation conditions. Furthermore, and regardless of the number of propagules, the outcome of the symbiosis depends on environmental factors, AM fungi characteristics and plant variables and our present knowledge makes it difficult to predict the effectiveness of the inoculum. It is not possible precisely to compare products based on monospecific cultures of the AM fungi to those based on mixtures. It is well known that growth dynamics of symbiotic associations differ significantly among AM fungi species. Some species can be regarded as more aggressive and fast growing while others are rather slow or their spores can be dormant, such as those AM fungi (despite infrequently used commercially) from *Gigaspora* and *Scutellospora* genera.

The procedure called “Direct Inoculum Production Process” (DIPP) could help to improve predictability of AMF inoculum effectiveness (Feldmann and Grotkass 2002). Nevertheless, a quality control of commercial inoculum must deal with several aspects as mentioned above. A reference system of information concerning AMF effectiveness based on the results of standard tests could be established.

Absence of microbial contaminants is vital issue mainly as regards international marketing. The use of good horticultural practice can avoid contamination and spread of unwanted (plant pathogens) microorganisms.

7 Intellectual Properties and their Effect on Market Development

Patents, if enforced, can act to inhibit market growth, or if licensed correctly could act to stimulate market growth.

Although patents cannot be secured for naturally-occurring organisms (as opposed to genetically manipulated organisms), application and manufacturing process patents can be obtained. In relation to mycorrhizal fungi, a series of the patents exists that protects, for example: specific cocktails of isolates for remediation effects, the use of rock wool in propagation systems, and the use of certain porous clays as substrates. Most of the patents currently operating in this field relative to existing production systems can be navigated around through different combinations of isolates and/or the addition of different additives in substrates. No significant patent actions have been noted in the market to date and it is generally believed, due to the vast amount of scientific publications supporting the field, that prior art could be discovered to inhibit any serious legal action.

The cost of patenting filing is relatively inexpensive, the cost of the associated national examination, translation and maintenance fees are though considerable. Accepting that much of the application of mycorrhizal fungi technology will be global, then worldwide filings would be needed to control a specific piece of intellectual property completely. As the returns from some markets will be modest, coupled with the cost of prosecution (and the lack of robustness of some court systems), it is unlikely that global patents will operate in this market.

It is also unlikely that new patenting will disturb the current industry, as the market can function, with good practice and an eye on reducing costs, using existing technology. The one area where intellectual property may act to affect the market development is in the field of in vitro culturing. Although some of the background for this work is in the public domain, there will be scope for controlling patents in this area. Who owns the relevant IPR and what strategy they will employ to exploit or control may have significant impact on the development of markets in certain countries.

8 Research that will Affect the Development of the Global Market for Mycorrhizal Fungi

Within the last couple of years, there has been a boom in commercial production and practical applications of mycorrhizal fungi inocula. Alongside this current market development, scientific evidence is building up more detailed knowledge on

various nutritional and nonnutritional effects of mycorrhizal fungi on fitness and health of individual plants, plant communities and soil environments.

All good research that discovers facts about the function of mycorrhizal fungi ultimately serves the market. A key area of research that will assist the current producers, who seek for commercial motives to stimulate the market, is the development of molecular probes for AMF. The classical way used to identify and evaluate AMF in roots and soil is not adapted for monitoring the inoculum efficiently. This is important for inoculum producers, as it will allow the identification and protection of their products as well as mapping their function as means to offering further evidence to the market of their efficacy. Also, the objections and questions aimed at possible invasiveness and competitive advantage will be more easily solved. More rapid and accurate methods, e.g., PCR-techniques, therefore need to be adapted to the demands of quality control, not only for AMF species to be detected but even strains and substrains. Several laboratories are working on this task, e.g., 'Generic' probes employing pPrimers based on LSU rDNA sequences and recognizing a wide range of Glomerales when used in nested PCR on soil and/or root DNA (Gollotte et al. 2004). These generic probes are useful for assessing the mycorrhizal status of soil and roots. 'Specific' probes have been developed in a pioneer work of van Tuinen et al. (1998), who proposed a method for characterizing root colonization profiles of AMF using 25S rDNA-targeted nested PCR of roots stained with Trypan blue allowing quantification of the AMF present. However, molecular probes defined at present are species-specific so that their practical use is limited to situations where a given inoculated AMF species is not yet present.

One of the important tasks of fundamental research is to get more information on how the AMF are becoming tolerant to certain environmental stresses (drought, soil contamination, pH fluctuations, etc.) and to what extent this tolerance can be stable through subculturing under stress-free conditions. It has been shown repeatedly that fungi can lose some of their functional features, e.g., tolerance to heavy metal throughout cultivation in heavy metal-free media (Malcova et al. 2002). There is always a risk of a selection of fungal strains under an optimized production system where there can be a shift of features originally possessed by particular strain. There is lack of knowledge on how changing conditions of subculturing affect symbiotic efficacy or adaptation of AMF strains. This knowledge is particularly vital for the use of inoculation in stressed or polluted soils where we should expect resistance of inoculants to particular types of stress.

As suggested previously, more knowledge on nonnutritional effects of mycorrhiza would be very significant for widening the market for inoculum producers. This includes the effects of mycorrhiza in production of essential oils by plants but also the role of mycorrhizal fungi in sequestering carbon in the soil. The latter is becoming of high commercial interest in relation to the establishment of bioenergetics plantations of mycorrhiza-dependent plants, mainly trees where possible trade of carbon sequestration quota might be considered.

Plant inoculation with more than one beneficial microbe type (plant biotisation) is an emerging technology and a new challenge for the industry of microbial

inocula and in particular for AMF inocula producers who could easily acquire such technology (Gianinazzi and Vosatka 2004). Inoculation using different combinations of beneficial rhizosphere microbes has shown how difficult it is to predict the outcome in terms of plant growth and health. Results vary according to the microbial combination used (Gianinazzi et al. 2003). Timing of inoculation may be crucial because, as pointed out by Barea et al. (2005), MF should play a key role in root morphology and functioning and therefore in the establishment of a microbial community. Significant increases in knowledge about soil microbes and microbial/plant interactions in the mycorhizosphere are necessary to choose suitable beneficial microbial combinations for inoculants and for optimizing the effects of AMF inoculation. This could open up to the mycorrhizal industry a new way for promoting a promising technology based on the multimicrobial inoculation, including a mycorrhizal component, and adding more unique selling points to the mycorrhizal market proposition.

9 Conclusions

The 'market makers' in the mycorrhizal fungi world are the companies that seek to make money from its exploitation, which is a basic rule of any business. Although many researchers may reflect poorly on this statement it is a matter of fact and if, as a scientific community, we seek to ensure that the technology is employed globally and to its best effect we need to support these commercial endeavours. Quality, regulation and product efficacy are all areas that need further focus and the employment of scientific expertise to help to accelerate mycorrhizal market development.

The producers have recognized that to ensure that their market is not damaged by the use of ineffective products, the quality is a virtue best driven from within. Coalitions of mycorrhizal inoculum producers have formed to develop self-imposed quality standards that both ensure best practice in production and also act as a barrier to the entry of other less conscientious producers. This is a positive step forward in the development of the mycorrhizal biotechnological sector and one that should also support any regulations that are imposed on the market. Product efficacy is still an area where further work is required. The appropriate dosage, or propagule density, for a given market sector is not yet formalized and it leaves scope for the marketers to set these values. Accepting that the development of DNA probes and the like will assist in quantifying efficacy limits, there are other approaches that the research community may wish to consider in the short-term to assist producers and the market in defining what should be the minimum treatment standard.

Many of the pioneer producers have developed domestic markets and are now developing together into a truly competitive industry involving worldwide spread of a mycorrhizal biotechnology market. Many of the experiences in marketing mycorrhizal products to the varied sectors are common among the producers. In this more competitive phase of the market development it will be interesting to

see how the management of these enterprises innovate to develop new markets and position themselves to secure market shares in developed markets.

If we accept that it is unlikely that funding will become available to support the mass application of mycorrhizal biotechnology in the third world, where there are numerous regions totally dependent on agriculture in arid or other degraded ecosystems, which in many cases are not sustainable, then our quest is to develop new business models that allow the industry to service this market oriented on benefits of a target country. Key to this goal is further research and innovation that will deliver lower-cost products that are traceable and are easy to manufacture and apply.

In conclusion, we can state that the mycorrhizal market is developing well and that it has excellent links between the research base and the producers, which are progressively tightening. We have some time to wait until mycorrhiza biotechnology becomes an integral part of good practice in global, sustainable horticulture and agriculture, but when it does it will deserve a title of truly green biotechnology for the third millennium. We dare to state that both the commercial and scientific communities are ready to promote this oncoming mycorrhizal age.

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References

Adriano DC, Wenzel WW, Vangronsveld J, Bolan NS (2004) Role of assisted natural remediation in environmental cleanup. Geoderma 122:121–142

Barea JM, Pozo MJ, Azcon R (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Brimner TA, Boland GJ (2003) A review of the non-target effects of fungi used to biologically control plant diseases. Agr Ecosyst Environ 100:3–16

Christie P, Li XL, Chen BD (2004) Arbuscular mycorrhiza can depress translocation of zinc to shoots of host plants in soils moderately polluted with zinc. Plant Soil 261:209–217

Dalpe Y (2005) Mycorrhizae: a potential tool for plant protection but not a panacea. Phytoprotection 86:53–59

Dassi B, Dumas-Gaudot E, Gianinazzi S (1998) Do pathogenesis-related (PR) proteins play a role in bioprotection of mycorrhizal tomato roots towards *Phytophthora parasitica*? Physiol Mol Plant P 52:167–183

Enkhtuya B, Oskarsson U, Dodd JC. (2003) Inoculation of grass and tree seedlings used for reclaiming eroded areas in Iceland with mycorrhizal fungi. Folia Geobot 38:2009–2022

Feldmann F, Grotkass C (2002) Directed inoculum production-shall we be able to design populations of arbuscular mycorrhizal fungi to achieve predictable symbiotic effectiveness? In: Gianinazzi S, Schuepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture, from genes to bioproducts. Birkhauser, Basel, pp 261–279

Feldmann F, Idczak E (1992) Inoculum production of vesicular-arbuscular mycorrhizal fungi for use in tropical nurseries. Method Microbiol 24:339–357

Gianinazzi S, Oubaha L, Chahbandar M, Blal B, Lemoine MC (2003) Biotization of microplants for improved performance. In: Proceedings of the XXVI International Horticultural Congress:

Biotechnology in Horticultural Crop Improvement: Achievements, Opportunities and Limitations. Acta Hortic 625:165–172
Gianinazzi S, Vosatka M (2004) Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. Can J Bot 82:1264–1271
Gianinazzi-Pearson V, Gianinazzi S, Trouvelot A (1985) Evaluation of the infectivity and effectiveness of indigenous vesicular-arbuscular fungal populations in some agricultural soils in Burgundy. Can J Bot 63:1521–1524
Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species Agrostis capillaris and Lolium perenne in a field experiment. Mycorrhiza 14:111–117
Gonzalez-Guerrero M, Azcon-Aguilar C, Mooney M, Valderas A, MacDiarmid CW, Eide DJ, Ferrol N (2005) Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Fungal Genet Biol 42:130–140
Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agr Ecosyst Environ 113:17–35
Graham JH, Miller RM (2005) Mycorrhizas: gene to function. Plant Soil 274:79–100
Greipsson S, El-Mayas H (1999) Large-scale reclamation of barren lands in Iceland by aerial seeding. Land Degrad Dev 10:185–193
Harrier LA, Watson CA (2003) The role of arbuscular mycorrhizal fungi in sustainable cropping systems. Adv Agron 79:185–225
Hawkes CV (2003) Nitrogen cycling mediated by biological soil crusts and arbuscular mycorrhizal fungi. Ecology 84:1553–1562
Hutchinson JJ, Young SD, Black CR, West HM (2004) Determining uptake of radio-labile soil cadmium by arbuscular mycorrhizal hyphae using isotopic dilution in a compartmented-pot system. New Phytol 164:477–484
Kotter J (1996) Leading change. Harvard Business School Press, Harvard University, Cambridge, Mass.
Janouskova J, Pavlikova D, Macek T, Vosatka M (2005) Arbuscular mycorrhiza decreases cadmium phytoextraction by transgenic tobacco with inserted metallothionein. Plant Soil 272:29–40
Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13
Liu R-J, Luo X-S (1994) A new method to quantify the inoculum potential of an arbuscular mycorrhizal fungi. New Phytol 128:89–92
Malcova R, Gryndler M, Vosatka M (2002) Magnesium ions alleviate the negative effect of manganese on *Glomus claroideum* BEG23. Mycorrhiza 12:125–129
Oliveira RS, Vosatka M, Dodd JC, Castro PML (2005) Studies on the diversity of arbuscular mycorrhizal fungi and the efficacy of two native isolates in a highly alkaline anthropogenic sediment. Mycorrhiza 16:23–31
Plenchette C, Clermont-Dauphin C, Meynard JM, Fortin JA (2005) Managing arbuscular mycorrhizal fungi in cropping systems. Can J Plant Sci 85:31–40
Puschel D, Rydlova J, Vosatka M (2008) Does the sequence of plant dominants affect mycorrhiza development in simulated succession on spoil banks? Plant Soil 302: 273-282
Rillig MC (2004) Arbuscular mycorrhizae, glomalin, and soil aggregation. Can J Soil Sci 84:355–363
Selosse MA, Baudoin E, Vandenkoornhuyse P (2004) Symbiotic microorganisms, a key for ecological success and protection of plants. C R Biol 327:639–648
Taylor J, Harrier LA (2003) Beneficial infuences of arbuscular mycorrhizal (AM) fungi on the micropropagation of woody and fruit trees. In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Kluwer, Dordrecht, pp 129–150
Tonin C, Vandenkoornhuyse P, Joner EJ, Straczek J, Leyval C (2001) Assessment of arbuscular mycorrhizal fungi diversity in the rhizosphere of Viola calaminaria and effect of these fungi on heavy metal uptake by clover. Mycorrhiza 10:161–168

van der Heijden MGA, Wiemken A, Sanders IR (2003) Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occurring plant. New Phytol 157:569–578

Van Tuinen D, Jacquot E, Zhao B (1998) Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. Mol Ecol 7:879–887

Vestberg M, Cassells AC, Schubert A, Cordier C, Gianinazzi S (2002) Arbuscular mycorrhizal fungi and micropropagation of high value crops. In: Giananazzi S, Schuepp H, Barea JM, Hasselwandter K (eds) Mycorrhizal Technology in Agriculture. Birkhauser, Basel, pp 223–233

Vosatka M (2001) A future role for the use of arbuscular mycorrhizal fungi in soil remediation: a chance for small-medium enterprises? Minerva Biotecnol 13:69–72

Vosatka M, Malcova R, Sramek F, Regvar M, Jansa J (1999) Inoculation with mycorrhizal fungi – a feasible biotechnology in horticulture. Phyton 3:219–224

Whipps JM (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bota 82:1198–1227

Zhang XH, Zhu YG, Chen BD, Lin AJ, Smith SE, Smith FA (2005) Arbuscular mycorrhizal fungi contribute to resistance of upland rice to combined metal contamination of soil. J Plant Nutr 28:2065–2077

Why and How Using Micropropagated Trees rather than Germinations for Controlled Synthesis of Ectomycorrhizal Associations?

Sylvie Herrmann(✉), François Buscot

1 Introduction

Mycorrhizal symbioses with soil fungi are observed on roots (or analogous organs) of more than 90% of the actual land plants (Smith and Read 1997), and there is fossil evidence that the mycorrhizal symbiosis already existed as plants started to colonize terrestrial habitats some 460 Mya (Redecker et al. 2000). The quasi-universality of mycorrhizas, their crucial ecological roles and practical importance in agriculture and forestry have prompted the development of more or less controlled culture systems enabling the synthesis of mycorrhizal associations for studying their formation, functions and effects (Declerck et al. 2005; Peterson and Chakravarty 1991; Wiemken 1994). Ideally, culture systems to synthesize mycorrhizas should use plant materials genetically defined and easy to produce at enough homogenously developed exemplars to allow statistical assessment of effects on plant performance or extraction of adequate amounts of metabolites or genetic material for functional analyses. Another positive trait is a short-term production system in order to reduce the delay of and between experiments.

In the case of ectomycorrhiza (EM), such expectations from controlled culture systems are more difficult for fulfil, as EM almost uniquely form on trees (Smith and Read 1997). Trees have a long life span during which they pass though highly different developmental and physiological stages. In addition, EM trees especially dominate in the boreal and temperate zones (Smith and Read 1997), which are marked by strong seasonal ecophysiological fluctuations. Provided seed dormancy can be broken down, numerous tree seedlings may be obtained rapidly, which constitute an easy to handle material for synthesizing EM due to their reduced size. However, from the requirements listed above, seedlings only partially fulfil the need for genetic homogeneity, and they do not necessarily develop synchronously. In addition seedlings are a very particular stage in plant life in terms of photosynthesis and internal energy

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budget. In developed plants, mycorrhizas are a sink of up to 20% of the net photo assimilation (Smith and Read 1997) therefore, culture systems using seedlings with weak photosynthesis may not be representative of the mycorrhizal function.

As an alternative to seedlings, tree cuttings or microcuttings can be used for synthesizing EM, which circumvents the genetic heterogeneity and the particular physiological state of plants with cotyledons. In 1985, Strullu pointed out that the quasi-totality of the in vitro synthesis of EM were realised with plantlets developed from surface sterilized seeds, although micro propagation of tree species was increasable accessible (Strullu 1985). Twenty years later, the situation has not really changed. Most recent cultivation-based studies on EM are still performed with seedling of pines or spruces (Bois et al. 2006; Van Hees et al. 2006; Wallander et al. 2006), oaks (Boling et al. 2006) or cedars (Boukcim et al. 2006).

With this background, the present chapter is devoted to explaining why using tree microcuttings in culture models to synthesize EM is advantageous. It also answers the question how, as it makes the point concerning difficulties in resolving the establishment of long-term tree clones. The first part of the chapter (Section 2) is devoted to a nonexhaustive review of some morphological and physiological traits that vary during tree development. These traits also have influence on EM formation and function as consequences for establishing tree microcutting clones, which is examined in Section 3. A third section (Section 4) explains the major technical steps for establishing long-term tree clones with a special focus on an exemplary model consisting in an oak clone (*Quercus robur* L.) that has been micropropagated for over 20 years to study EM formation, physiology and function. After some concluding remarks (Section 5), a brief outlook (Section 6) mentions the perspective that tree genetic transformation opens in this research field.

2 Variations in Morphology, Physiology and Architecture during Tree Development, Consequences on EM Symbiosis and Culture Models for Their Study

This Section does not aim at making an exhaustive review on tree development but only to point to major differences between seedlings and older plants of trees especially in terms of C resource allocation. It also mentions some traits in development physiology and architecture that change during the long life span of trees and that have an impact for the producing and the function of tree microcuttings, and which are the backbone of the further Sections.

2.1 Cotyledons, Primary Leaves, Adult Leaves: Morphology and Physiology

During their development from seeds through juvenile seedlings to adults, trees develop different types of leaves. The greatest difference is observed between cotyledons and all further leaf types.

In seeds of most plants, cotyledons store substances such as proteins, carbohydrates or fats, which are mobilized during germination and supply the resources for a heterotrophic life stage until the seedling has developed its photosynthetic apparatus and become autotrophic (Schöpfer and Brennecke 2006). Impact of seed size was studied in large-seeded tree species for which reserves in cotyledons play a determining role for germination and early growth of young seedlings. Intraspecific variations in seed sizes correlate with embryonic soluble sugars (Merouani et al. 2003), root growth parameters (McPhee 2005) and development of primary leaves (Ichie et al. 2001). Such differences make it difficult to optimize a regular production of homogeneously-developed plants to routinely make EM synthesis assays.

Work performed on herbaceous plants, which are easier to handle than trees, has shown that the average rate of net photosynthesis in mature cotyledons almost entirely supports the dry mass production of young seedlings and is much higher than the photo assimilation of the primary leaves that develop at this stage (Jucknischke and Kutschera 1998). Gehring (2004) also showed that interspecific seed size variations play a determining role in the ecological performance of tropical trees growing under light-limited understory of rain forests.

After germination, all the leaf types produced by trees during their life span are not equivalent at the morphological and physiological levels. One year and older seedlings often differ drastically concerning their phyllotaxy and leaf morphology (Kozlowski 1971). Furthermore, tree size (seedling compared to canopy trees) is an important factor influencing foliar gas exchange and water relationships (Fredericksen et al. 1996). In *Quercus rubra*, different drought responses were observed between seedlings, which close their stomata early in the day at the expense of C uptake, and mature trees that are able to access deeper water reserves and to adjust their intrinsic water use efficiency (Cavender-Bares and Bazzaz 2000). For the rain-forest tree *Macaranga gigantea*, Ishida et al. (2005) also reported lowered intrinsic water-use efficiency and photochemical capacity of photosystem II in leaves of seedlings, and could relate leaf morphology and physiology to differences in resource use at each life stage. Seedlings of some species (such as pines) produce primary leaves that are physically and physiologically distinct from both cotyledons and mature leaves. Photosynthesis and stomata conductance are reduced in aging trees (Bond 2000; Kostner et al. 2002; Rust 2002), but also with the size of the tree (Mencuccini 2007).

Differential functioning in leaves of juvenile and mature trees has also been investigated at the gene expression level (Woo et al. 1994). Earlier work on *Cynara scolymus L.* (Moncousin and Gaspar 1983) had pointed out a relationship between peroxidase activity of juvenile trees and their rooting potential (Pierik 1990).

All these facts underline the fact that seedlings represent a very particular stage in tree life from an energetic point of view.

2.2 Ontogeny, Tree Architecture and Rhythmic Growth

Architecture models of trees as described by Hallé et al. (1978) are mostly related to the growth dynamic of the trees. Many trees of temperate regions which form mycorrhiza show episodic growth, e.g., *Quercus robur, Pinus pinaster, Acer pseudoplatanus,*

Fraxinus excelsior, Abies alba, Picea abies, Castanea sativa, Ilex aquifolium. In the special case of the *Fagaceae*, differences between seedlings and adult trees in the development mode and the form of the branches are more particularly observed for chestnut and beech species but less so in oaks (Soumoy et al. 1996). All species show an orthotropic growth during the first year. In the following years, the orthotropic growth is maintained in oaks and gives the architecture model of Rauh, whereas for chestnuts a mix of plagiotropic lateral branches and orthotropic principal sprouts, and for beeches only plagiotropic lateral and principal sprouts, develop giving, respectively, the two models of Massart and Troll. Determination of the rhythmic growth was studied for tropical and temperate trees (Barnola et al. 1990; Champagnat et al. 1986; Edelin 1981; Hallé and Martin 1968; Millet et al. 1991). Maintenance of endogenous rhythmic growth under culture conditions was demonstrated for *Erica* (Beaujard and Astié 1983), *Quercus* (Favre and Juncker 1989) and *Hevea* (Lardet et al. 1998).

Root architecture is often dependent on the provenance of the plants. Mulatya et al. (2002) noticed more shallow rooting for cuttings than for seedlings. Similar observations were made in oaks, for which marked differences were found between the root architecture of seedlings (Lavarenne 1968; Belgrand et al. 1987; Harmer 1990) and of cuttings (Riedacker and Belgrand 1983). In oak seedlings, the root system developed from large acorns presents a typical herringbone pattern with an orthogeotropic taproot and semi-plagiotropic lateral roots (Lavarenne 1968), reflecting the strong hierarchy between the linear growing tap root and the lateral roots with limited growth. In cuttings, architecture of root systems reflects a lower hierarchy, which is strongly influenced by the origin and the physiological age of the cuttings (Riedacker and Belgrand 1983).

Independently of their architecture, root systems of trees are characterized by a dimorphism between short and long or mother roots, which is called heterorhizis. The two root types differ in their absorption and anchorage functions and present characteristic differences in their anatomy (Strullu 1985). Short roots have a limited lifetime and are developed on mother roots. Both types of roots are able to form EM. More generally, however, mycorrhizas are formed on short roots and present a limited viability. Anatomical differences in ectomycorrhizas related to this heterorhizis were described in *Eucalyptus* (Massicotte et al. 1987), *Fagus* (Clowes 1951) and *Pinus* (Wilcox 1968; Robertson 1954).

3 Tree Cuttings and Microcuttings for Controlled Synthesis of EM Symbiosis

3.1 Amelioration in Comparison to Seedlings

Apart the already mentioned advantages in terms of genetic homogeneity and suppression of the cotyledon effects, the use of cuttings or microcuttings avoids difficulties related to obtaining sterile germinations. Most culture models with

tree seedlings proceed to seed sterilization for enhancing the chance to synthesize EM with the inoculated fungal partner. Small seeds such as the ones of pines or eucalypts are easy to sterilize with hydrogen peroxide, $HgCl_2$, or calcium hypochlorite (Kottke et al. 1987; Wong and Fortin 1988). In contrast, large seeds such as oak acorns are more difficult to disinfect completely (Herrmann et al. 1992).

3.2 Cuttings and Microcuttings for EM Formation

Willows are easy to propagate under in situ conditions as cuttings, and this cloned material has been used in EM research. For different clones of such cuttings of *Salix repens* L., the impact of the plant origin on effectiveness of EM symbiosis could be determined precisely (Van der Heijden and Kuyper 2001). *Salix viminalis* cuttings were also used to study the allocation of ^{14}C in EM (Durall et al. 1994). For many trees however, cutting regeneration does not work and an in vitro procedure multiplying aseptic microcuttings has to be employed.

Although tissue culture techniques have been applied for plant regeneration and micropropagation since the mid-1930s, this approach was considered as extremely difficult during succeeding decades for woody plants displaying marked physiological differences between juvenile and adult trees and with strong inhibition of dormant buds (Champagnat 1983). Routine propagation of trees from the genera *Malus* (Druart et al. 1992), *Prunus* (Riffaud and Cornu 1981), *Erica* (Beaujard and Astié 1983), *Pinus* (Rancillac 1979) or *Picea* (Misson et al. 1982) was however established in the 1980s. Micropropagation of *Quercus robur* followed few years later (Chalupa 1984; Favre and Juncker 1987; Meier-Dinkel 1987; Vieitez et al. 1985).

In vitro synthesis of EM on micropropagated trees was attempted as soon as the technique of in vitro propagation was established for trees (Strullu et al. 1984). The main goal of these initial works with EM fungi was to trigger the rooting difficulties encountered with in vitro propagated shoots, especially when they were gained from physiologically mature stock trees. At this time, EM fungi were known for their stimulation of short root formation during formation of mycorrhizas, which led Slankis (1973) to formulate his theory about the implication of fungal auxins in the EM symbiosis establishment. Many investigations on in vitro tree cloning with mycorrhization were presented for diverse angiosperms, such as birches (Grellier et al. 1984), chestnut (Strullu et al. 1986), oaks (Favre et al. 1987) or ash (Grange et al. 1997; Simoneau et al. 1994; Strullu et al. 1984), and also for gymnosperms like pines (David et al. 1979; Rancillac 1982) or spruce (Stein et al. 1988).

The list of successful long-term micropropagation and rooting of trees is getting more and more exhaustive. Nevertheless, systems using such micropropagated trees for current EM symbiosis studies are still largely underrepresented and limited to a few species such as poplars (Kaldorf et al. 2002; Marjanovic et al. 2005; Nehls

2007) or birches (Simoneau et al. 1993; 1994) for the species without episodic growth, and species such as oaks (Buscot and Herrmann 2004; Frettinger et al. 2007; Krüger et al. 2004; Herrmann et al. 1998, 2004; Herrmann and Buscot 2007) or pine (Normand et al. 1996; Niemi et al. 2002) for trees with episodic growth. The scarce literature available over the last decades on using cuttings or micropropagated trees in the field of EM research in comparison to the numerous recent studies that use tree seedlings (see Introduction) gives further evidence for this discrepancy.

3.3 Micropropagation Steps and Difficulties to Overcome

For micropropagation of trees, differences between episodic and non-episodic growing trees have to be considered (McCown and McCown 1987). Woody perennial plants, for which the shoot growth is characterized by strong episodic flushes during the growing season, are often extremely difficult to micropropagate. Such plants often maintain their episodic development under the in vitro culture conditions, and tend to degenerate during the resting periods that alternate with periods of shoot growth. In particular, highly episodic growing trees such as oaks (*Quercus*), walnuts (*Juglans*), spruces (*Picea*) or firs (*Abies*) have rarely been successfully micropropagated when starting from older stock plants and are still difficult to keep in long-term culture even when the stock plants are seedlings not older than 2 years. Episodic growing species contrast markedly with species that show continuous shoot growth through the growing seasons. Therefore, most successful trees used in microculture and biotechnology are non-episodic growing tree species such as poplars (*Populus*), elms (*Ulmus*), eucalypts (*Eucalyptus*) and birches (Betula).

Prior to micropropagation of recalcitrant seedlings or older trees, a rejuvenation of the stock plant by serial grafting or cutting cycles is most needed (Evers et al. 1993; Greenwood 1987; Hackett 1985; Mc Cown 2000; Vermeer 1991; Vieitez et al. 1994; Zsaczek et al. 2006). However, despite preliminary rejuvenation, microcuttings keep in their memory the physiological age of the original stock plant, which can be expressed for example in a plagiotropic growth or in a total absence of rooting (Fouret et al. 1984; Franclet et al. 1987; Franclet and Franclet-Mirvaux 1992; Pierik 1990; Vermeer 1991). The loss of orthotropic morphogenesis renders such microcuttings unsuitable as they fail to develop into vigorous trees. Rooting of micro cuttings from older stock seedlings (up to 2 or 3 years) can be successfully induced in some cases under ex vitro conditions with high humidity (Meier-Dinkel et al. 1993), but in vitro adventitious rooting is often only possible after an in vitro rejuvenation obtained by repeated subcultures (Chabukswar and Deobhar 2006; Hackett 1985; McCown 2000) or by adding growth substances (Monteuuis and Bon 2000; Pierik et al. 1997; Stromquist and Hansen 1980) or activated charcoal (Dumas and Monteuuis 1995; Favre and Juncker 1987).

4 Micropropagation of an Episodic Growing Tree: The Oak Model (*Quercus robur* L.)

Trees of the genus *Quercus* have monopodial orthotropic trunks and branches that display a rhythmic growth, thus following the architectural model of Rauh described by Hallé et al. (1978). Champagnat et al. (1986) and Lavarenne (1966) showed that under controlled culture conditions (i.e., 25°C and long day illumination) the endogenous rhythmic growth is expressed by the development of successive growth units or modules with a periodicity of 21 days. The seasonal climates in combination with the tree age are responsible for a reduction to 1–5 growth units within a vegetation period (Gruber 1987; Lavarenne 1966). In trees older than 10 years, the number of growth flushes is mostly reduced to two: one in spring and a so-called "Johannistrieb" growth unit in august. Under extreme climatic conditions repeated "Johannistrieb" can be observed. Growth units are characterized by a typical heteroblastie with successive formation of scale leaves, photosynthetic leaves and leaves with aborted blade (Hallé and Martin 1968). Each growth unit (or module) is characterized by an acrotonic lateral branching, in which the most apical axillaries buds give rise to vigorous ramification (Harmer 1989).

The establishment of in vitro shoot propagation involves successive stages: (1) the primary culture, (2) the stabilization and long-term culture, and (3) the rooting and acclimatization of the plants.

4.1 Primary Cultures

The primary cultures are established from axillary or apical nodal explants. After suppression of the apical dominance, inhibiting effects on lateral buds outgrowth are broken (Jacobs et al. 1959; Wang and Wareing 1979; Wickson and Thimann 1958). This suppression of the inhibiting correlations allows an expression of the morphogenetic potentialities of isolated lateral buds. Internal hormonal balances between cytokinins and auxins determine the outgrowth of each nodal explant and these balances vary according to several factors:

- *Age of the stock plant*. Success in establishing primary cultures depends greatly on the age of the oak trees, and for older individuals a forced rejuvenation by cutting regeneration is often needed prior to the establishment of an in vitro culture (Evers et al. 1993; McCown 2000; Vieitez et al. 1994). Lignification generally lowers outbursting of explanted buds, and clones gained from such buds often display a reduced rooting capacity (Juncker and Favre 1989).
- *Genetic origin*. The genetic origin of the oak clone affects the shoot growth, the multiplication factor and the number of possible subcultures.
- *Initial bud position*. As mentioned above, growth units (modules) of intact plants are acrotone segments in which the upper buds first develop, while lower buds are repressed. For primary culture, nodal explants can be taken from seedlings

or young shoots over several successive growth units. Morphogenetic gradients along the stem and various growth responses of the explants are observed in relation to the initial position of the lateral buds from which they are obtained. Juncker and Favre (1994) noticed this influence and used only basal nodes for micropropagation as these produced longer shoots with more numerous leaves. San-Jose et al. (1988) observed that the percentage of explants forming shoots in all clones was greater for apical than for nodal culture and the number of shoots per explant was greater for nodal than for apical culture. As the origin of the material used for the different experimentations was quite different, generalization of observations made on the position effects of the explants on the in vitro development of cuttings remains difficult. Developmental differences between apical and nodal stem sections also depend on whether the shoots used are under elongation or resting at the time of the excision (Puddephat et al. 1997). Explants excised from elongating shoots perform poorly, and this observation has also been made when in vitro cultivated shoots at the elongation stage were used for propagation. A basipetal increase in morphogenic response was observed also in vitro, which was most pronounced during the early developmental stage (Volkeart et al. 1990). The frequency of the shoot development and subsequent formation of multiple shoot is also influenced by the number of buds. Explants forming basal and apical regions with multiple axillary buds produced the lowest frequencies of developing shoot explants, while single axillaries excised from the midstem positions gave the greatest amount of developing shoot explants (Puddephat et al. 1997).

- *Nutrient and hormonal composition of the culture media*. The effects of mineral nutrients and growth regulators composition on primary culture establishment of oak are well documented (Meier-Dinkel 1987; Chalupa 1984; Vieitez et al. 1985; Favre and Juncker 1987, 1989). With explants from young seedlings, optimal primary cultures were obtained with 0.1 mg/l of the cytokinin 6-benzyl aminopurine (BA) and 30 g/l sucrose on the MS/2 (NN/4) medium in which concentration of NH_4NO_3 is reduced to a quarter compared to the original Murashige and Skoog medium (Favre and Juncker 1987, 1989). The beneficial effect of BA at 1 mg/l was confirmed by Puddephat et al. (1987). Addition of BA is also recommended in other tree micropropagation procedures (David et al. 1979; Riffaud and Cornu 1981).

Expression of episodic growth during primary in vitro culture was observed by Favre and Juncker (1987) and suggests that growth in explants remains dominated by endogenous rhythmic cycles. This trait is typical of species where growth occurs by extension of preformed buds (McCown and McCown 1987). Favre and Juncker (1989) described variations in the expression of the rhythmic growth mode of oak microcuttings in relation to the composition of the culture medium and defined three in vitro growth patterns: an episodic or rhythmic growth, a prolonged growth and a continuous growth. The episodic or rhythmic growth with formation of successive time- and size-limited growth units is only obtained on medium without BA and in the presence of activated charcoal, corresponding to the conditions that allow

spontaneous or induced rooting of the explants. The prolonged growth corresponds to the formation of a single flush with production of more regular internodes and more numerous assimilating leaves. This second pattern was the most often observed under normal propagation conditions, that is only the addition of BA. Shoots with a prolonged growth could be successfully subcultured but never showed spontaneous rooting. To a limited extent, a third growth pattern with continuous growth was described with a continuous production of regular internodes and leaves. This pattern is still expressed in two or three further subcultures but is generally followed by degeneration of the clone due to apical necrosis. Such continuous growth pattern is mostly induced on N-rich media.

4.2 Stabilization and Long-Term Culture

As for primary cultures, genetic origin plays a determining role in the establishment of a long-term culture (Chalupa 1988; Juncker and Favre 1989; Meier-Dinkel et al. 1993). In many cases, new developed shoots degenerated after a successful primary culture or even after several subcultures for no apparent reason. In other cases, a progressive degeneration can occur at a time at which long-term culture is occurring. After 6–7 months, the clonal effect is the most important determining factor of the behavior of a long-term culture (Juncker and Favre 1994).

In the following, we will focus on presentation of results obtained with a single oak clone (the juvenile clone DF 159), which was established in Nancy (France) by Bertrand Juncker in 1986 (see publications with this author's name). The clone was kindly given to our research group in autumn 1990 where it is still propagated today.

4.2.1 Culture Media Used: Combined Influence of Nutrient and Hormonal Composition

Over the last 15 years, the culture media used have been adapted in their nutrient composition and hormonal supply. An optimal total N concentration was essential to avoid degeneration of the oak clone (Juncker and Favre 1994). For this reason, we used two basic media: the MS half-strength culture medium with 1/8 or 1/4 NH_4NO_3 and the modified GD Medium as proposed by Juncker and Favre (1994) (Gresshoff and Doy 1972; Murashige and Skoog 1962; and Table 1).

In order to define the best hormonal application for maintaining long-term propagation, we observed, over several years, the behavior of the microcuttings under diverse combinations of the auxin indole-3-butyric acid (IBA) and the cytokinin 6-benzyl aminopurine (BA). Finally combinations between nutrient and hormonal supply had to be regularly adapted over the years according to the plant growth response. Concerning the hormones, we opted for two solutions: BA used alone at a concentration of 0.1 mg/l and a combined application of BA and IBA with

Table 1 Culture media for oak propagation and rooting

	Propagation medium MS[a]/2 NH_4NO_3/4		Propagation medium GD[b]		Rooting medium GD[b]/2	
Macronutrients (mg/l)	KH_2PO_4	85	$NaH_2PO_4 \cdot H_2O$	90	$NaH_2PO_4 \cdot H_2O$	45
			$Na_2HPO_4 \cdot 2H_2O$	30	$Na_2HPO_4 \cdot 2H_2O$	15
	KNO_3	950	KNO_3	1,000	KNO_3	500
	NH_4NO_3	412	$(NH_4)_2 \cdot SO_4$	200	$(NH_4)_2 \cdot SO_4$	100
	$CaCl_2 \cdot 2H_2O$	225	$CaCl_2 \cdot 2H_2O$	150	$CaCl_2 \cdot 2H_2O$	75
			KCl	300	KCl	150
	MgSO4·7H_2O	185	$MgSO_4 \cdot 7H_2O$	250	$MgSO_4 \cdot 7H_2O$	125
Micronutrients[a] (mg/l)	H_3BO_3	6.2	H_3BO_3	6.2	H_3BO_3	6.2
	$MnSO_4 \cdot H_2O$	16.9	$MnSO_4 \cdot H_2O$	16.9	$MnSO_4 \cdot H_2O$	16.9
	$ZnSO_4 \cdot 7H_2O$	10.59	$ZnSO_4 \cdot 7H_2O$	10.59	$ZnSO_4 \cdot 7H_2O$	10.59
	KI	0.83	KI	0.83	KI	0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.25	$Na_2MoO_4 \cdot 2H_2O$	0.25	$Na_2MoO_4 \cdot 2H_2O$	0.25
	$CaCl_2 \cdot 6H_2O$	0.025	$CaCl_2 \cdot 6H_2O$	0.025	$CaCl_2 \cdot 6H_2O$	0.025
	$CuSo_4 \cdot 5H_2O$	0.025	$CuSo_4 \cdot 5H_2O$	0.025	$CuSo_4 \cdot 5H_2O$	0.025
Fe-EDTA[a] (g/l)	Na_2-EDTA	37.2	Na_2-EDTA	37.2	Na_2-EDTA	37.2
	$FeSO_4 \cdot 7H_2O$	27.8	$FeSO_4 \cdot 7H_2O$	27.8	$FeSO_4 \cdot 7H_2O$	27.8
Vitamins + Organic Compounds[b] (mg/l)	Thiamin	1	Thiamin	1	-	
	Pyridoxin	0.1	Pyridoxin	0.1	-	
	Nicotinic acid	0.1	Nicotinic acid	0.1	-	
	Glycin	0.4	Glycin	0.4	-	
	Myo-inositol	40	Myo-inositol	40	-	
	Glutamine	40	Glutamine	40	-	
Sucrose (g/l)	30		30		20	
Agar[c] (g/l)	6		6		6	
Activated charcoal[d] (g/l)					1	
pH	5.6		5.6		5.6	

[a]Composition after Murashige and Skoog (1962)
[b]Composition after Gresshof and Doy (1972)
[c]From Serva 11396
[d]From Merck 2186

identical concentrations of 0.2 mg/l. BA alone was used for intensive shoot multiplication and BA+IBA was used for vigorous shoot growth before root induction. BA+IBA was also tested over 2 years at a double concentration (0.4 mg/l). During this time, we examined the incidence of doubled growth substances application during propagation on rooting (see below and Fig. 2). In order to keep the practical procedure as simple as possible, not more than 2 of 4 possible combinations between the two basic culture media: MS/2 (with total N reduced to 1/8 and later on to 1/4) and GD, and the two hormonal supplies (BA alone or BA+IBA) were used in alternation at the same time.

4.2.2 Growth Patterns Observed During Long-term Micropropagation: Relation to the Initial Position of the Explants Within the Growth Unit

Two growth patterns were observed during long-term propagation. A first pattern, characteristic of an expression of the endogenous rhythmic growth, presented a growth flush with long internodes and scaled leaves at the basis of the stem followed distally by short nodes with a small number of normal leaves with varying blade size (Fig. 1d). The second growth pattern was more typical for the prolonged growth also described by Favre and Juncker (1989). Here, stems were thicker with regular internodes separating smaller numerous leaves of homogenous size (Fig. 1b).

These two patterns were observed on all media tested even if their expression was slightly modulated depending on the hormonal supply. From previous works, we know that a growth unit is a complex entity expressed in the heteroblastie coupled with internodes of different sizes (Champagnat et al. 1986; Hallé and Martin 1968). As these dual morphogenetic patterns were expressed in vitro,

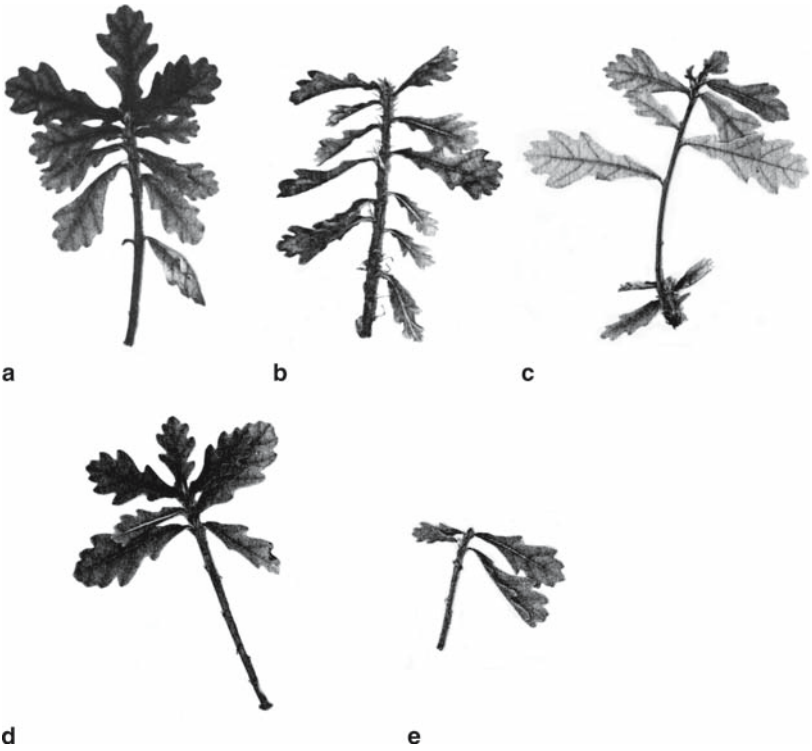

Fig. 1 Expression of rhythmic or prolonged growth patterns during oak micropropagation after subculture from apical explants (**a, b, c**) or from axillary explants **(d, e)**

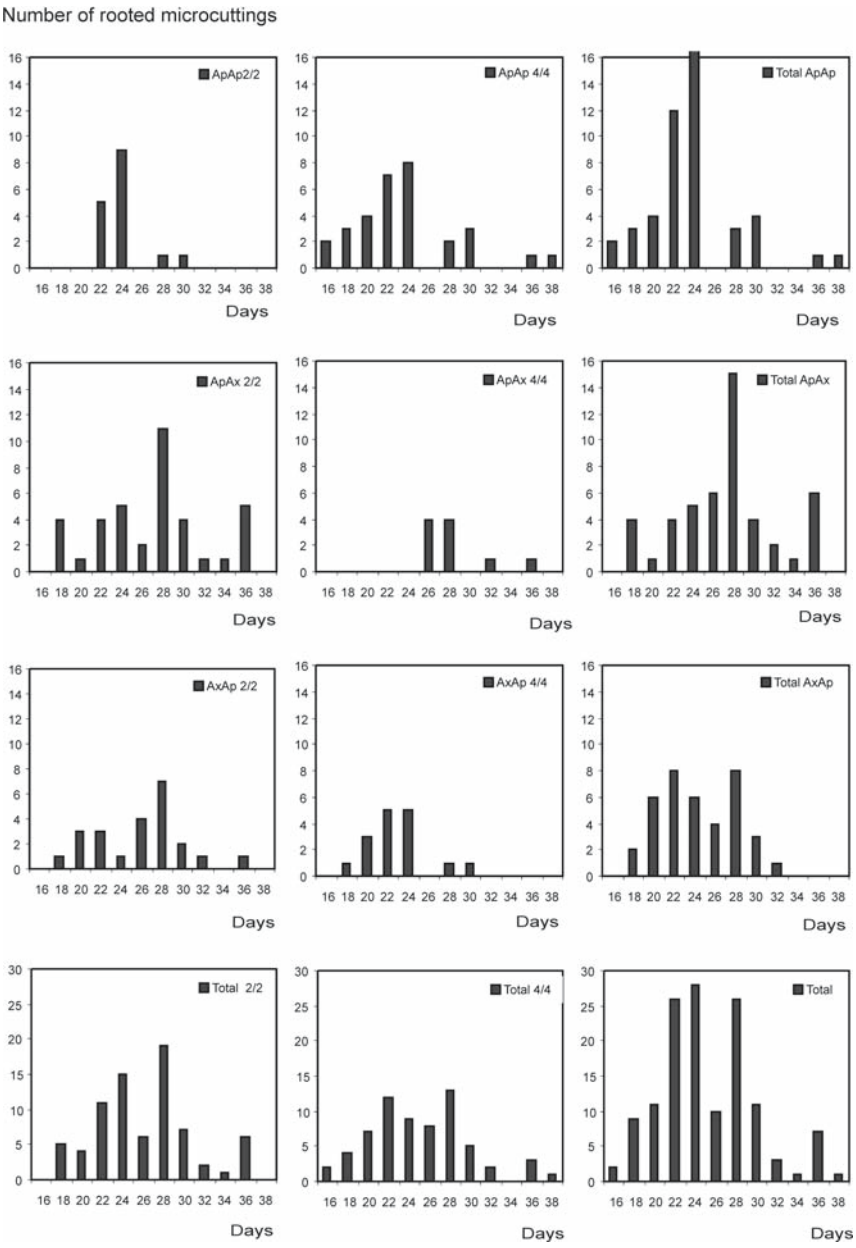

Fig. 2 Rooting delay after transfer on activated charcoal rooting medium. Incidence of two hormonal concentrations 0.2 mg/l or 0.4 mg/l for combined use of BA and IBA (2/2 and 4/4) during plant development before root induction and of the initial position of the explants over the two last subcultures (*ApAp*, *ApAx* and *AxAp*)

Table 2 Development of apical and axillary shoots on MS/2 propagation medium with BA and IBA at 0.2 mg/l. Internode size is up to 2 mm for small internodes, between 2 and 4 mm for intermediary and over 4 mm for large internodes

	Apical nodes (Ap)		Axillary nodes (Ax)
	Apical shoots	Axillary's shoots	Axillary's shoots
Number of shoots	16	23	76
Shoot length (mm ± SE)	33.6 ± 2.0 a	20.3 ± 1.6 b	21.5 ± 0.9 b
Thick stemmed shoots	100%	17.4%	75%
Shoots with:			
- small internodes	1	0	0
- intermediary internodes	15	6	13
- long internodes	0	17	62
	Rhythmic or prolonged growth	Rhythmic growth	Rhythmic growth

a, b Indicates significantly different at $P \leq 0.05$

despite correlative inhibitions between apical and lateral buds on the very first mother plant having been suppressed for a long time, we hypothesized that they could be related to the position from which explants are taken on the microcuttings within the growth unit at each subculture step.

In fact, on culture media with combined BA and IBA, we observed that shoots developed from apical explants and from axillary explants showed differing morphologies (Table 2). The apices of apical explants developed in thick-stemmed shoots with internodes of intermediary size and were able to develop both growth patterns. In contrast, shoots developed from axillary explants were characterized by predominantly long internodes and had only a rhythmic growth pattern (Fig. 1d, e). Axillary shoots developed from apical explants showed also a rhythmic growth but were more fragile (Fig. 1c).

4.2.3 How to Warrant Long-term Production of High Quality Microcuttings?

Long-term propagation has a double goal: intensive multiplication of shoots and production of vigorously developing plants for rooting, which should display as much morphogenetic similarity to modules on adult oak trees for reliable experimentation. From Favre and Juncker (1987, 1989), we know that, during primary culture, rhythmic growing shoots present the highest rooting ability, and we postulated that this feature should also be maintained during long-term propagation even if it is known that frequent subcultures induce rejuvenation and enhance the rooting ability. In the following experiments, we examined the incidence of bud position effect during subcultures performed on two alternate culture media in order to orientate the subcultures toward multiplication or production of vigorous shoots for rooting.

Experiment with BA Alone and Alternation of Apical (Ap) and Axillary (Ax) Propagation to Promote Efficient Multiplication

Propagation was performed on a GD medium + BA 0.1 mg/l. Morphogenetic potentialities of the buds were examined considering the axillary or apical original position of the explants and the level at which the axillary explants were cut off. Responses were also compared between equivalent explants sampled along apical (Table 3) versus axillary shoots (Table 4). Explants sampled on apical shoots showed higher survival and propagation rates compared to explants from axillary shoots. Basal axillary nodes with scarred leaves and long internodes developed stronger shoots (increased leaf area, increased shoot length, increased proportion of thick stemmed shoots) (see Tables 3 and 4) than upper axillary nodes with normal leaves and short internodes. This heterogeneous shoot development was similarly

Table 3 Development of apical (*Ap*) and axillary (*Ax*) shoots on GD propagation medium with BA 0.1 mg/l. Propagation is performed from explants of apical shoots. Axillary explants are numbered acropetally from 1 to 3

	ApAp Apical nodes		AxAp 1 Distally axillary nodes with normal leaves separated by short internodes	AxAp 2 Intermediary axillary nodes with normal leaves separated by long intenodes	AxAp 3 Proximally axillary nodes with scarred leaves
Number of explants	47		47	46	52
Survival rate	85%		100%	98%	78%
Number of developed shoots	41		54	57	45
Propagation rate	1.02		1.15	1.24	0.86
Leaves area (mm^2 ± SE)	136 ± 8 b		137 ± 8 b	186 ± 11 a	207 ± 10 a
	Apical shoots	Axillary shoots	Axillary shoots	Axillary shoots	Axillary shoots
Number of shoots	13	17	54	57	45
Shoot length (mm ± SE)	162 ± 11 b	180 ± 9 ab	164 ± 8 b	209 ± 10 a	216 ± 10 a
Thick stemmed shoots	92.3%	11.0%	17.6%	9.3%	44%
Shoots with:					
- small internodes	13	1	17	13	4
- intermediary internodes	0	16	17	24	19
- long internodes	0	0	0	6	13
	Prolonged growth	Rhythmic growth	Rhythmic and prolonged growth	Rhythmic and prolonged growth	Rhythmic and prolonged growth

a, b Indicates significantly different at $P \leq 0.05$

Table 4 Development of apical (*Ap*) and axillary (*Ax*) shoots on GD propagation medium with BA 0.1 mg/l. Propagation is performed from explants of axillary shoots. Axillary explants are numbered acropetally from 1 to 3

	ApAx Apical nodes		AxAx 1 Distally axillary nodes with normal leaves	AxAx 2 Proximally axillary nodes with scarred leaves
Number of explants	65		47	47
Survival rate	84.6%		31.9%	44.7%
Number of shoots	55		15	23
Propagation rate	0.85		0.32	0.49
Leaves area (mm^2 ± SE)	164 ± 13 ab		138 ±16 b	197 ± 17 a
	Apical shoots	Axillary's Shoots	Axillary's shoots	Axillary's Shoots
Number of shoots	17	15	15	23
Shoot length (mm ± SE)	214 ± 19 a	220 ± 24 a	168 ± 13 b	209 ± 17 a
Thick stemmed shoots	53%	40%	0%	38%
Shoots with				
- small internodes	8	0	10	4
- intermediary internodes	9	12	5	8
- long internodes	0	3	0	8
	Prolonged growth	Rhythmic and prolonged growth	Rhythmic and prolonged growth	Rhythmic and prolonged growth

a, b Indicates significantly different at $P \leq 0.05$

expressed in shoots developed form axillaries sampled along apical shoots (AxAp1, AxAp2, AxAp3) and along axillary shoots (AxAx1, AxAx2). If we consider the total explants manipulated in the experience, we can conclude that over two subcultures the best responses are obtained by alternating the origin of the explants (see AxAp or ApAx in Tables 3 and 4).

Experiment with BA and IBA and Repeated Sequences of Ap and Ax Subcultures to Promote Development

In a further set of experiments, we examined the impact of repeated sequences of apical (Ap) or axillary (Ax) multiplication over more than 2 subcultures. The used medium was MS/2 + BA 0.2 mg/l + IBA 0.2 mg/l. In general, the shoots developed on this medium were more vigorous than those developed on GD + BA. When propagation was performed exclusively from axillary explants, only a very few rhythmic growing shoots with extremely thin stems developed soon after the third consecutive axillary subculture (AxAxAx) (Figs. 1e and 3a2). If propagation was restricted to apical explants, the developed shoots became progressively thicker,

with extremely reduced internodes and small leaves of equal size. The shoot size was mostly reduced to less than 1 cm after four subcultures (ApApApAp) and axillary shoots developed on such shoots, indicating a reduction of the apical dominance (Fig. 1c). These two extreme situations were not interesting to consider for production of vigorous shoot within long-term propagation, but demonstrate the high difference of morphogenetical potentialities related to the original position of the explants and the possibility to induce the degeneration of a clone if subcultures are restricted to one type of explants. To avoid this degeneration and find the best adapted sequences between apical and axillary subculture for production of vigorous rhythmic growing shoots, different sequences of alternating propagation from axillary and apical nodes were performed over three subcultures and observed (Table 5). Survival and propagation rate are maximal for apical explants obtained after maximal two consecutive subcultures with identical explants origin (see Table 5, ApApAx or ApAxAp). The shoots developed under these conditions present also the highest vigour (highest shoot length, leaf area, stem thickness, rhythmic growth) (Figs. 1a and 3-a2).

In all experiments we observed that longest shoot length is obtained on BA + IBA medium from nodes of different initial positions (Tables 2 and 5). Mean length above 30 mm, which is required for further experimentation with rooted shoots, is obtained for apical shoots developed on apical explants (Ap in Table 2 and ApApAx in Table 5). This size was also reached for shoots developed from apical shoots originating from intermediary or basal axillary explants (ApAxAp 2+3). The last situation illustrates the basitonie of the growth units, which is maintained over two subsequent subcultures. With a third subculture of apical explants (ApApAp), shoot elongation is reduced. Longest shoot length is noticed for shoots after two subsequent axillary subcultures, but in this case only fragile shoots develop. Optimal shoot length, thickness and rhythmic growth are obtained for ApApAx or ApAxAp when the Ax propagation was performed in previous subculture from the shoot base (Table 5).

4.3 Rooting and Acclimatisation

As previously mentioned, rooting is the most delicate phase during tree micropropagation. Adventitious rooting of micropropagated plants needs an induction to trigger initiation and development of roots that require exogenous factors such as an auxin treatment, some hormonal inhibitors or activated charcoal. For oaks, material of adult origin shows a clonal-specific effect on the rooting capacity of the microcuttings (Junker and Favre 1989, 1994; Meier-Dinkel 1987). In contrast, rapid repeated subculturing of microcuttings appears to provide an intrinsic stimulus for an adult-to-juvenile transition leading to a better rooting ability (McCown 2000). In particularly difficult situations and in order to simplify the protocol, ex vitro rooting can by apply. However, ex vitro rooting requires in vitro shoots of high quality (Meier-Dinkel et al. 1993). In vitro rooting of oaks was performed by

Table 5 Development of apical and axillary shoots on MS/2 propagation medium with BA and IBA at 0.2 mg/l

	ApApAp		**ApApAx**		**ApAxAp 1* ApAxAp (2+3)°**		**AxAxAp 1* AxAxAp (2+3)°**	**AxAxAx**
Number of explants	19		19		21* 53°		18* 49°	18
Survival rate	89%		100%		100%* 79%°		41%* 48%°	8%
Number of developed shoots	17		19		21* 42°		8* 31°	3
Propagation rate	0.89		1.0		1.00* 0.79°		0.44* 0.63°	0.17
Leaves area (mm^2 ± SE)	146.9 ± 4.9 a		92.9 ± 2.4 b		130.6 ± 15.7° b		161.5 ± 5.4° a	
	Apical shoots	Axillary shoots	Apical shoots	Axillary shoots	Apical shoots	Axillary shoots	Axillary shoots	Axillary shoots
Number of shoots	6	11	3	16	20* 40°	1* 2°	8* 31°	3
Shoot length (mm ± SE)	256±25b'	235±20b'	330±26a'	346±14a'	264 ± 14* b 358 ± 15° a		360 ± 42* a 376 ± 13° a	
Thick stemmed shoots	100%	91%	100%	87%	71%* 92%°		0%* 0%°	
Shoots with:								
- small internodes	3	0	1	0	12* 11°	0* 0°	0* 0°	
- intermediary internodes	3	11	20	12	9* 28°	0* 0°	0* 0°	
- long internodes	0	0	0	2	0* 1°	0* 2°	31* 8°	
	Prolonged and rhythmic growth		Rhythmic growth		Rhythmic growth		Rhythmic growth	

* Indicates shoot issue from distally Ax explants; ° indicates proximally Ax explants as indicated in Table 3
a, b and a', b' Indicate significantly different at $P \leq 0.05$

Fig. 3 Microcuttings developed on MS/2 with BA and IBA using alternating apical and axillary node subculture or no more than two consecutive subcultures with identical origin (**a1**) and after repeated subcultures from axillary explants (**a2**). Ramified root system developed on GD/2 + activated charcoal medium (**b**)

Junker (personal communication) on two successive media: a first one for induction with half-strength nutrient (GD/2 Medium), lower sucrose concentration and auxin, and a second one for root development where auxin is replaced by activated charcoal at 1 g/l medium and sucrose concentration reduced to 20 g/l. By selecting vigorous shoots characterized by a rhythmic growth, it was possible to reach a high percentage of rooted shoots though using a unique culture medium, i.e., GD half-strength with activated charcoal at 1 g/l (Herrmann et al. 1998). In this procedure, the most vigorous shoots that had accomplished their first growth flush were sorted and the callus tissue formed around the cut wound was gently pruned off. Rooting was also stimulated by increasing the light intensity (Herrmann et al. 1998).

For synthesis and study of ectomycorrhizal symbiosis, synchronously rooted plants are basically required (Herrmann et al. 2004). A further optimization of the procedure therefore consisted in synchronizing the rooting delay. During experiments

performed in the last two decades we carefully noted the rooting delays and examined the impact of all crucial modifications occurring during micropropagation. In particular, we examined the influence of doubling the hormonal supply and of the initial position of the buds taken as explants for the micropropagation (Fig. 2). Normally, the first tips of newly developed adventitious roots become visible in the cultivation tubes 16–18 days after transfer onto the rooting medium with activated charcoal mentioned above (Fig. 2). Pronounced maximal formation of visible roots is reached after 22–24 days and a further peak occurs 1 week later, i.e. after 28 days. A two-fold concentration of BA + IBA during the last subculture before rooting induction did not appear to modify this typical rooting delay profile, and in the cumulative graphs, no marked peak indicating a narrower rooting time window was observed (compare Total 2/2 and Total 4/4 in Fig. 2). On the contrary, rooting delays were markedly influenced by alternating propagation of apical and axillaries for the last two subcultures prior to rooting induction. Narrower rooting time peaks were notable after 22 and 24 days for ApAp and after 28 days for ApAx, respectively (compare Total ApAp and Total ApAx in Fig. 2). These observations point out how important it is to consider the history of micropropagated shoots used for rooting. If we sort plants by taking into account their morphological characteristics, we are able to produce homogenous developed plants with synchronous rooting. In such homogenous vigorous plants, the root system starts to ramify into secondary roots 12 days after formation of the first roots, which coincides with a development phase at which the apical buds reach their out bursting stage (Fig. 3b) (Herrmann et al. 1998).

4.4 Microcuttings and Aseptic Ectomycorrhizal Synthesis

EM synthesis if often performed in semi-sterile Petri-dish systems where only the shoot develops ex-vitro, while the roots are kept under aseptic conditions (Herrmann et al. 1998; Kottke et al. 1987; Wong and Forting 1988). For such culture systems, rooted oak microcuttings at the apical node bursting stage just described are very adequate plant material. The formation of a new shoot growth unit immediately after transfer onto the Petri-dish system largely contributes to a successful acclimatizing to the semi-sterile conditions.

For the purpose of experiments on EM synthesis in which two biological partners are involved, it appeared essential to strictly control the shoot production. Therefore, at each step of the microculture we optimized the propagation procedure. Not only for establishing primary cultures (Favre and Juncker 1987), but also during long-tem propagation and rooting, it was important to permanently adjust external factors such as light or the composition of culture media. In addition, it was essential to consider the initial position of the bud explants, which determines endogenous specific morphogenetic potentialities. Integration of these external factors and endogenous conditioning along the long-term micropropagation of our oak clone can be resumed in tracing the history of the produced shoots. By using microcuttings of similar topological origin it is possible to link the plant development towards a synchronous rooting

and mycorrhization. Micropropagated oak series obtained in that way have a comparable homogenous vigor and growth pattern at the beginning of experiments. With such plant material, synchronous development can be linked further on, once the transfer to the mycorrhiza synthesis system has occurred. Such homogenous plants provided the basis for fine physiological (Herrmann et al. 2004) and transcriptomic studies (Krüger et al. 2004; Frettinger et al. 2006, 2007).

5 Conclusions

In this chapter, we have described the use of microcuttings of trees in studies based on EM synthesis. We especially made the point that apart the advantage of using a clonal material, the procedure enables one to get small rooted plant modules without the special impact of cotyledons, for example on the energy budget, which have developmental traits encountered in adult trees. In particular, a long-term in vitro micropropagated oak clone made it possible to consider for the first time the impact of the episodic growth of major forest trees on their mycorrhization under controlled aseptic conditions. In our long-term clone, the endogenous rhythmic growth is expressed in vitro and the shoot and root flushes are developed out of phase (Herrmann et al. 1998). With a lower hierarchy between principal roots and second order roots, the rooted microcuttings have a root system which is more comparable to that of cuttings or older trees than to that of seedlings (Herrmann and Buscot 2007). Indeed, our miniaturized oak cuttings simulate a functional module of an adult tree, and this enabled us to compare mycorrhizal dynamics between fungi with variable carbon demand, for example, *Paxillus involutus* versus *Piloderma croceum* (Herrmann et al. 1998). Following this line, it was possible to analyze the competition for nutrient allocation between lateral root elongation and mycorrhizal formation in diverse fungal partners and especially in *Pisolithus tinctorius* versus *Lacaria amethystea* (Buscot and Herrmann 2004). With both fungi, the impact of the episodic shoot and root growth on mycorrhization dynamics was demonstrated. With the presented optimized Petri-dish system using oak microcuttings the opportunity was also presented to study the impact of heterorhizie on mycorrhization at a molecular level. Differential gene regulation patterns were found in principal and lateral roots (Frettinger et al. 2007; Herrmann and Buscot 2007).

6 Outlook

During tree ontogeny, variations in morphological traits reflect the strong physiological changes that occur from seedling through nonreproductive stages to flowering adult trees. In tree cuttings (grafting) and in microcuttings, expression of traits typical for already developed trees is maintained. The arguments listed in Section 2 largely argue for the necessity to consider the impact of different ontogenetic and physiologic

stages during the tree life span in the framework of research on EM. However, to our knowledge, a strict comparison between EM colonization on seedlings, cuttings and microcuttings has not so far been performed, although statistical tools would allow differentiating between effects related to the genetic heterogeneity within seedlings and their different physiology compared to cuttings and microcuttings.

Additional arguments for considering the impact of tree ontogenetic aspects in controlled experiments with EM are given by the numerous ecological studies, which largely document colonization by EM fungi differs between seedlings, and old stand trees. Rao et al. (1997) made the point that *Boletus luteus, Scleroderma aurantium, Tricholoma saponaceum*, and *Hygrophorus limacinus* were early-colonizing EM fungi on *Pinus kesiy*, while in older plantations, late-stage EM with *Russula lepida* and *Amanita phalloides* were observed as late-stage fungi. Furthermore, species richness increases with stand age (Dighton and Mason 1985). Early- and late-stage EM fungi were shown to have different carbohydrates demands (Mason and Last 1986), and it is generally admitted that fungi with low nutrient demands form EM on younger trees and vice versa. A high dependence to glucose for the late stage colonizer *Amanita muscaria* during EM formation was, for example, reported by Gibson and Deacon (1990), who found EM formation by this species to be triggered by increased glucose concentration. The use of well-characterized microcuttings expressing specific ontogenetic traits would enable a better control of experimental conditions with adequate numbers of replicates in that research field. The impact of complex parameters involved in ecological mycorrhizal successions could be more easily disentangled.

A last reason to consider micropropagated trees in the field of EM research is related to the increasing progresses in the field of tree genetic transformation (Pena and Seguin 2001). Transformation systems of poplar species (Fillatti et al. 1987; Leple et al. 1992) have been available for 20 years and the first studies on EM of transformed poplars have been published (Kaldorf et al. 2002). With the more recent transformation of larch (Gleeson et al. 2005) and birch (Vauramo et al. 2006), but also of episodic growing trees like pines (Tang et al. 2001) or oak (Alvarez et al. 2004), the number of transformable trees is increasing. For the model tree oak, embryogenesis from tissues of mature oak trees (*Quercus suber*) is possible (Valladares et al. 2006; Lopes et al. 2006). The technique may be extendable to other *Quercus* species and offers an access to regeneration and selection of transformed oak trees. This opens a perspective to make regulation studies of genes that have been found to be specifically expressed at certain stages of plant ontogeny (Krüger et al. 2004) or on certain root types (Frettinger et al. 2007) during EM interaction, and to consider the impact of root rhythmic growth displayed by adult trees and microcuttings but not similarly by seedlings.

References

Alvarez R, Alonso P, Cortizo M, Celestino C, Hernandez I, Toribio M, Ordas R (2004) Genetic transformation of selected mature cork oak (*Quercus suber* L.) trees. Plant Cell Rep 23:218–223

Barnola P, Alatou D, Lacointe A, Lavarenne S (1990) Etude biologiques et biochimiques du déterminisme de le croissance rythmique du chêne pédonculé (*Quercus robur* L.). Effects de l'ablation des feuilles. Ann Sci For 21:619–631

Beaujard F, Astié M (1983) Les Bruyères in vitro. I. Culture *in vitro* de L'Erica x darleyensis et mise en évidence d'une croissance rythmique accentuée des rameaux orthotropes. Can J Bot 61:3533–3535

Belgrand M, Dreyer E, Joannes H, Velter C, Scuiller I (1987) A semi-automated data processing system for root growth analysis: application to a growing oak seedling. Tree Physiol 3:393–404

Bois G, Bigras FJ, Bertrand A, Piché Y, Fung MYP, Khasa DP (2006) Ectomycorrhizal fungi affect the physiological responses of *Picea glauca* and *Pinus banksiana* seedlings exposed to an NaCl gradient. Tree Physiol 26:1185–1196

Boling BC, Naab FU, Smith D, Duggan JL, McDaniel FD (2006) Leaf element analysis in mycorrhizal post oak seedlings. Nucl Instr Method 251:182–190

Bond BJ (2000) Age-related changes in photosynthesis of woody plants. Trends Plant Sci 5:349–353

Boukcim H, Pagés L, Mousain D (2006) Local NO3- or NH4+ supply modifies the root system architecture of *Cedrus atlantica* seedlings grown in a split-root device. J Plant Physiol 163:1293–1304

Buscot F, Herrmann S (2004) At the frontier between basidiomycotes and plants: reciprocal interactions between mycorrhiza formation and root development in an in vitro system with oaks and hymenomycetes. In: Agerer R, Piepenbring M, Blanz P (eds) Frontiers in basidiomycote mycology. IHW, Eching, pp 361–376

Cavender-Bares J, Bazzaz FA (2000) Changes in drought response strategies with ontogeny in *Quercus rubra*: implications for scaling from seedlings to mature trees. Oecologia 124:8–18

Chabukswar MA, Deodhar MA (2006) Restoration of rooting competence in a mature plant of *Garcinia indica* through serial shoot tip grafting in vitro. Sci Hortic 108:194–199

Chalupa V (1984) *In vitro* propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* MILL.). Biol Plant 26:374–377

Chalupa V (1988) Large scale micropropagation of oak (*Quercus robur* L.) using adenine-type cytokinins and thidiazuron to stimulate shoot proliferation. Biol Plant 30:414–421

Champagnat P (1983) Quelques réflexions sur la dormance des bourgeons des végétaux ligneux. Physiol Vég 21:607–618

Champagnat P, Payan E, Champagnat M, Barnola P, Lavarenne S, Bertolon C (1986) La croissance rythmique de jeunes chênes pédonculés cultivés en conditions contrôlées et uniformes. Naturalia Monspeliensia – Colloque International sur l'Arbre, Montpellier, pp 303–337

Clowes FAL (1951) The structure of mycorrhizal roots of *Fagus sylvatica*. New Phytol 50:1–16

David A, David H, Faye, M. Isemukali K (1979) Culture *in vitro* et micropropagation du Pin maritime (*Pinus pinaster* sol.) AFOCEL, Etud Rech 12:33–40

Declerck S, Strullu DG, Fortin JA (2005) *In vitro* culture of mycorrhizas. In: Varma A (ed) Soil biology, vol 4. Springer, Heidelberg, pp 341–375

Dighton J, Mason PA (1985) Mycorrhizal dynamics during forest tree development. In: Moore D, Casselton LA, Woods DA, Frankland JC (eds) Development biology of higher fungi. Cambridge University Press, Cambridge, pp 117–139

Druart P, Klevers C, Boxus P, Gaspar T (1982) In *vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Z Pflanzenphysiol 108:429–436

Dumas E, Monteuuis O (1995) *In vitro* rooting of micropropagated shoots juvenile and mature *Pinus pinaster* explants: influence of activated charcoal. Plant Cell Tissue Organ Cult 40:231–235

Durall DM, Jones MD, Tinker PB (1994) Allocation in C-14 carbon in ectomycorrhizal willow. New Phytol 128:109–114

Edelin C (1981) Some features of conifers vegetative architecture. Lett Bot 128:177–188

Evers P, Vermeer E, Van Eeden S (1993) Rejuvenation of *Quercus robur*. Ann. Sci. Forestière 50:330–335

Favre JM, Dexheimer J, Juncker B, Lei J (1987) Vitroplants et mycorhization controlée: deux outils complémentaires pour valoriser du matériel de qualité un exemple: *Quercus robur* L. Actes du 2. Colloque sciences et industries du bois, Nancy, pp 185–192

Favre JM, Juncker B (1987) *In vitro* growth of buds taken from seedlings and adult plant material in *Quercus robur* L. Plant Cell Tissue Organ Cult 8:49–60

Favre JM, Juncker B (1989) Variations in expression of episodic growth by in vitro cultured shoots of oak (*Quercus rubur* L.). Ann Sci For 46:206–210

Fillatti JJ, Sellmer J, McCown B, Haissing B, Comai L (1987) *Agrobacterium*-mediated transformation and regeneration of *Populus*. Mol Gen Genet 206:192–199

Fouret Y, Arnaud Y, Larrie C (1984) Rajeunissement *in vitro* du *Sequoia sempervirens*. AFOCEL Annales des recherches sylvicoles, pp 111–137

Franclet A, Boulay M, Ekkaoui F, Fouret Y, Verschoore-Martouzet B, Walker N (1987) Rejuvenation. In: Bonga M, Durzan DJ (eds) Cell and tissue culture in forestry, vol. 1 General principles and biotechnology. Nijhoff, Dordrecht, pp 232–248

Franclet A, Franclet-Mirvaux MC (1992) Elimination of topophysis in *Sequoia sempervirens* - clone NP29: A model plant useful to progress in understanding the concept of rejuvenation. Proceedings of the IUFRO Symposium "Mass production technology for genetically improved fast growing forest tree species", Bordeaux, AFOCEL, Nangis, pp, 113–125

Fredericksen TS, Steiner KC, Skelly JM, Joyce BJ, Kolb TE, Kouterick KB, Ferdinand JA (1996) Diel and seasonal patterns of leaf gas exchange and xylem water potentials of different-sized *Prunus serotina* Ehrh trees. For Sci 42:359–365

Frettinger P, Herrmann S, Lapeyrie F, Oelmüller R, Buscot F (2006) Diffferential expression of two class III chitinases in two types of roots of *Quercus robur* during pre-mycorrhizal interactions with *Piloderma croceum*. Mycorrhiza 16:219–223

Frettinger P, Derory J, Herrmann S, Plomion C, Lapeyrie F, Oelmüller R, Martin F, Buscot F (2007) Transcriptional changes in two types of pre-mycorrhizal roots and in ectomycorrhizas of oak microcuttings inoculated with *Piloderma croceum*. Planta 225:331–340

Gehring CA (2004) Seed reserves and light intensity affect the growth and mycorrhiza development of the seedlings of an Australian rain-forest tree. J Tropic Ecol 20:345–349

Gibson F, Deacon JW (1990) Establishment of ectomycorrhizas in aseptic culture: effects of glucose, nitrogen and phosphorus in relation to successions. Mycol Res 94:166–172

Gleeson D (2005) Overproduction of proline in transgenic hybrid larch (*Larix leptoeuropaea* Dengler) cultures renders them tolerant to cold, salt and frost. Mol Breed 15:21–29

Grange O, Bärtschi H, Gay G (1997) Effect of the ectomycorrhizal fungus *Hebeloma cylindrosporum* on *in vitro* rooting of micropropagated cuttings of arbuscular mycorrhiza forming *Prunus avium* and *Prunus cerasus*. Trees 12:49–56

Greenwood MS (1987) Rejuvenation of forest trees. Plant Growth Regul 6:1–12

Grellier B, Letouzé R, Strullu DG (1984) Micropropagation of birch and mycorrhizal formation *in vitro*. New Phytol 97:591–570

Gresshof OM, Doy CH (1972) Development and differentiation of haploid *Lycopersicum esculentum* (tomato). Planta 107:161–170

Gruber F (1987) Über die sylleptische Verzeigung der Johannistriebe von Rotbuche und Stieleiche. Allg Forst Z 49:1283–1285

Hackett WP (1985) Juvenility, maturation, and rejuvenation in woody plants. Hortic Rev 7:109–155

Hallé F, Martin R (1968) Etude de la croissance rythmique chez l'hévéa (*Hevea brasiliensis* Müll.-Arg. euphorbiacées-crotonoidées). Adansonia:475–503

Hallé F, Oldeman RA, Tomlinson PB (1978) Tropical trees and forests: an architectural analysis. Springer, Heidelberg

Harmer R (1989) The effect of mineral nutrients on growth, flushing, apical dominance and branching in *Quercus petraea* (Matt.) Liebl For Oxford 62:383–395

Harmer R (1990) Relation of shoot growth phases in seedling oak to development of the tap root, lateral roots and fine root tips. New Phytol 115:23–27

Herrmann S, Buscot F (2007) Cross talks at the morphogenetic, physiological and gene regulation levels between the mycobiont *Piloderma croceum* and oak microcuttings (*Quercus robur* L.) during formation of mycorrhizas. Phytochemistry 68:52–67

Herrmann S, Ritter T, Kottke I, Oberwinkler F (1992) Steigerung der Leistungsfähigkeit von Forstpflanzen (*Fagus silvatica* L. und *Quercus robur* L.) durch kontrollierte Mykorrhizierung. Allg Forst Jagdz 4:72–79

Herrmann S, Munch JC, Buscot F (1998) A gnotobiotic culture system with oak microcuttings to study specific effects of mycobionts on plant morphology before, and in the early phase of ectomycorrhiza formation by *Paxillus involutus* and *Piloderma croceum*. New Phytol 138:203–212

Herrmann S, Oelmüller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between oak microcuttings and *Piloderma croceum*: influence on plant development and photosynthesis. J Plant Physiol 161:509–517

Ichie T, Ninomiya I, Ogino K (2001) Utilization of seed reserves during germination and early seedling growth by *Dryobalanops lanceolata (Dipterocarpaceae)*. J Trop Ecol 17:371–378

Ishida A, Yazaki K, Hoe AL (2005) Ontogenetic transition of leaf physiology and anatomy from seedlings to mature trees of a rain forest pioneer tree, *Macaranga gigantea*. Tree Physiol 25:513–522

Jacobs WP, Danielson J, Hurst V, Adams P (1959) What substance normally controls a given biological process? 2. The relation of auxin to apical dominance. Devel Biol 1:534–554

Jucknischke A, Kutschera U (1998) The role of the cotyledons and primary leaves during seedling establishment in sunflower. J Plant Physiol 153:700–705

Juncker B, Favre JM (1989) Clonal effects in propagating oak trees via *in vitro* culture. Plant Cell Tissue Organ Cult 19:267–276

Juncker B, Favre JM (1994) Long-term effects of culture establishment from shoot-tip explants in micropropagating oak (*Quercus robur* L.). Ann Sci For 51:581–588

Kaldorf M, Fladung M, Muhs HJ, Buscot F (2002) Mycorrhizal colonization of transgenic aspen in a field trial. Planta 214:653–660

Kostner B, Falge E, Tenhunen JD (2002) Age-related effects on leaf area/sapwood area relationships, canopy transpiration and carbon gain of Norway spruce stands (*Picea abies*) in the Fichtelgebirge, Germany. Tree Physiol 22:567–574

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An *in vitro* method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Kozlowski TT (1971) Growth and development of trees. I. Seed germination, ontogeny and shoot growth. Academic, New York

Krüger A, Peskan T, Frettinger P, Herrmann S, Buscot F, Oelmüller R (2004) Identification of pre-mycorrhiza related plant genes in the association between *Quercus robur* and *Piloderma croceum*. New Phytol 163:149–157

Lardet L, Aguilar ME, Michaux-Ferriere N, Berthouly M (1998) Effect of strictly plant-related factors on the response of *Hevea brasiliensis* and *Theobroma cacao* nodal explants cultured *in vitro*. In Vitro Cell Dev Biol Plant 34:34–40

Lavarenne S (1966) Croissance rythmique de quelques espèces de chênes cultivés en conditions climatisées. C R Acad Sci Ser III 262:358–361

Lavarenne S (1968) Croissance comparée des tiges et des racines de jeunes chênes cultivés en conditions contrôlée. C R Acad Sci Ser III 266:778–780

Leple JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L (1992) Transgenic Poplars – expression of chimeric genes using 4 different constructs. Plant Cell Rep 11:137–141

Lopes T, Pinto G, Loureiro J, Costa A, Santos C (2006) Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiol 26:1145–1152

Marjanovic Z, Uehlein N, Kaldenhoff R, Zwiazek JJ, Weiss M, Hampp R, Nehls U (2005) Aquaporin in poplar: What a difference a symbiont makes! Planta 222:258–268

Mason PA, Last FT (1986) Are the occurrence of sheathing mycorrhizal fungi in new and regenerating forests and woodlands in Scotlands predictable. In: Jenkins D (ed) Trees and wildlife in Scottish uplands. Institute of Terrestrial Ecology, Huntingdon, pp 63–70

Massicotte HB, Peterson RL, Ashford AE (1987) Ontogeny of *Eucalyptus pilularis-Pisolithus tinctorius* ectomycorrhizae. I. Light microscopy and scanning electron microscopy. Can J Bot 65:1927–1939

McCown BH (2000) Special symposium: *in vitro* plant recalcitrance. Recalcitrance of woody and herbaceous perennial plants dealing with genetic predeterminism. In Vitro Cell Dev Biol Plant 36:149–154

McCown DD, McCown BH (1987) North American hardwoods. In: Bonga JM, Durzan DJ, (eds) Cell and tissue culture in forestry, vol 3. Case histories: gymnospermes, angiosperms and palms. Nijhoff, Dordrecht, pp 247–260

McPhee K (2005) Variation for seedling root architecture in the core collection of Pea Germplasm. Crop Sci 45:1758–1763

Meier-Dinkel A (1987) *In vitro* propagation and *in vitro* establishment of pedunculate oak (*Quercus robur* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.). Allg Forst Jagdz 158:199–204

Meier-Dinkel A, Becker B, Duckstein D (1993) Micropropagation and ex vitro rooting of several clones of late-flushing *Quercus robur* L. Ann Sci For 50:319–322

Mencuccini M, Martinez-Vilalta J, Hamid HA, Korakaki E, Vanderklein D (2007) Evidence for age- and size-mediated controls of tree growth from grafting studies. Tree Physiol 27:463–473

Merouani H, Apolinario LM, Almeida MH, Pereira JS (2003) Morphological and physiological maturation of acorns of cork oak (*Quercus suber* L.). Seed Sci Technol 31:111–124

Millet B, Bonnet B, El-Morsy AW (1991) Le fonctionnement rythmique des végétaux ligneux. L'arbre: biologie et développement, pp 295–317

Misson JP, Coumans M, Giot-Wirgot P, Gaspar T (1982) Induction de bourgeons adventifs sur bourgeons de *Picea pungens* en culture *in vitro*. Z Pflanzenphysiol 107:161–167

Moncousin C, Gaspar T (1983) Peroxidase as a marker for rooting improvement of *Cynara scolymus* L. cultured in vitro" Biochem Physiol Pflanzen 178:263–271

Monteuuis O, Bon MC (2000) Influence of auxins and darkness on in vitro rooting of micropropagated shoots from mature and juvenile *Acacia mangium*. Plant Cell Tissue Organ Cult 63:173–177

Mulatya JM, Wilson J, Ong CK, Deans JD, Srent JI (2002) Root architecture of provenances, seedlings and cuttings of *Melia volkensii*: implications for crop yield in dryland agroforestry. Agrofor Syst 56:65–72

Murashige, T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:1327–1334

Nehls U (2007) Mastering mycorrhizal symbiosis: Sugar as signal and nutrient in *Amanita muscaria*/poplar ectomycorrhizas. Comp Biochem Physiol A 146:216–216

Niemi K, Häggman H, Sarjala T (2002) Effects of exogenous diamines on the interaction between ectomycorrhizal fungi and adventitious root formation in Scots pine in vitro. Tree Physiol 22:373–381

Normand L, Bärtschi H, Debaud J-C, Gay G (1996) Rooting and acclimatization of micropragated cutting of *Pinus pinaster* and *Pinus sylvestris* are enhanced by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. Physiol Plant 98:759–766

Pena L, Seguin A (2001) Recent advances in the genetic transformation of trees. Trends Biotechnol 19:500–506

Peterson RL, Chakravarty P (1991) Techniques in synthesizing mycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 23. Harcourt Brace Jovanovich, London, pp 75–106

Pierik RLM (1990) Rejuvenation and micropropagation. Progress in plant cellular and molecular biology. Proc VIIth Int Cong Plant Tissue and Cell Culture, Amsterdam, pp 91–101

Pierik RLM, Oosterkamp J, Ebbing MAC (1997) Factors controlling adventitious root formation of explants from juvenile and adult *Quercus robur* fastigiata. Sci Hortic 71:87–92

Puddephat IJ, Alderson PG, Wright NA (1997) Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro*. J Exp Bot 48:951–962

Rancillac M (1979) Morphologie du système racinaire de *Pinus pinaster* Sol. sur bourgeons isolés ou plantes entières *in vitro*: mycorhization. C R Congr Nat Soc Sav 104:377–389

Rancillac M (1982) Multiplication végétative *in vitro* et synthèse mycorhizienne: pin maritime - Hebelome, Pisolithe. Les mycorrhizes: biologie et utilisation. Les Colloques de l'INRA 13:351–356

Rao CS, Sharma GD, Shukla AK (1997) Distribution of ectomycorrhizal fungi in pure stands of different age groups of *Pinus kesiya*. Can J Microbiol 43:85–91

Riedacker A, Belgrand M (1983) Morphogénèse des systèmes racinaires des semis et boutures de chêne pédonculé. Plant Soil 71:131–146

Redecker D, Kodner R, Graham LE (2000) Glomelean fungi from the Ordovician. Science 289:1920–1921

Riffaud JL, Cornu D (1981) Utilisation de la culture *in vitro* pour la multiplication de Merisiers adultes (*Prunus avium* L.) selectionnés en forêt. Agronomie 1:633–640

Robertson NF (1954) Studies on the mycorrhiza of *Pinus sylvestris*. New Phytol 53:253–283

Rust S, Roloff A (2002) Reduced photosynthesis in old oak (*Quercus robur*): the impact of crown and hydraulic architecture. Tree Physiology 22:597–601

San-Jose MC, Ballester A, Vieitez AM (1988) Factors affecting in vitro propagation of *Quercus robur* L. Tree Physiol 4:281–290

Schöpfer P, Brennecke A (2006) Pflanzenphysiologie 6. Auflage. Elsevier, München

Simoneau P, Viemont JD, Moreau JC, Strullu DG (1993) Symbiosis-related polypetides associated with early stages of ectomycorrhiza organogenesis in birch (*Betula pendula* Roth). New Phytol 124:495–504

Simoneau P, Juge C, Dupuis JY, Viemont JD, Moreau C, Strullu DG (1994) Protein biosynthesis changes during mycorrhiza formation in roots of micropropagated birch. Acta Bot Gal 141:429–435

Slankis V (1973) Hormonal relationships in mycorrhizal development. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizae. Academic, New-York, pp 231–298

Smith JE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Soumoy L, Thiébaut B, Sérey I (1996) Développement de quelques Fagaceae japonaises; germination, modèle architectural, et systématique. Can J Bot 74:1248–1259

Stein A, Fortin JA, Vallee G (1988) Enhanced rooting of *Picea mariana* cuttings by ectomycorrhizal fungi. Can J Bot 68:468–470

Stromquist L, Hansen J (1980) Effects of auxin and irradiance on the rooting of cuttings of *Pinus sylvestris*. Physiol Plant 49:346–350

Strullu DG (1985) Les mycorrhizes. Borntraeger, Berlin

Strullu DG, Grellier B, Letouzé, R (1984) Mycorhizes et micropropagation *in vitro*: concepts et réalisations. C R Acad Agri Fr 70:1331–1337

Strullu DG, Grellier B, Marciniak D, Letouzé R (1986) Micropropagation of chestnut and conditions of mycorrhizal synthesis *in vitro*. New Phytol 102:95–101

Tang W, Sederoff R, Whetten R (2001) Regeneration of transgenic loblolly pine (*Pinus taeda* L.) from zygotic embryos transformed with *Agrobacterium tumefaciens*. Planta 213:981–989

Valladares S, Sanchez C, Martinez MT, Ballester SA, Vieitez AM (2006) Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RADP analysis. Plant Cell Rep 25:879–886

Van der Heijden EW, Kuyper TW (2001) Does origin of mycorrhizal fungus or mycorrhizal plant influence effectiveness of the mycorrhizal symbiosis? Plant Soil 230:161–174

Van Hees PAW, Rosling A, Essens S, Godbold DL, Jones DL, Finlay RD (2006) Oxalate and ferricrocin exudation by the extramatrical mycelium of an ectomycorrhizal fungus in symbiosis with *Pinus sylvestris*. New Phytol 169:367–377

Vauramo S, Pasonen HL, Pappinen A, Setala H (2006) Decomposition of leaf litter from chitinase transgenic silver birch (*Betula pendula*) and effects on decomposer populations in a field trial. Appl Soil Ecol 32:338–349

Vermeer E (1991) Rejuvenation, micropropagation and field testing of *Quercus robur*. Acta Hortic 289:324–325

Vieitez AM, San-Jose MC, Vieitez E (1985) In vitro plantlet regeneration from juvenile and mature *Quercus robur*, L. J Hortic Sci 60:99–106

Vieitez AM, Sanchez M, Amo-Marco J, Ballester A (1994) Forced flushing of branch segments as a method for obtaining reactive explants of mature *Quercus robur* trees for micropropagation. Plant Cell Tissue Organ Cult 37:287–295

Wallander H, Hagerberg D, Aberg G (2006) Uptake of Sr-87 from microcline and biotite by ectomycorrhizal fungi in a Norway spruce forest. Soil Biol Biochem 38:2487–2490

Wang TL, Wareing PF (1979) Cytokinins and apical dominance in *Solanum andigena*: lateral shoot growth and endogenous cytokinin levels in the absence of roots. New Phytol 82:19–28

Wickson M, Thimann KV (1958) The antagonism of auxin and kinetin in apical dominance. Physiol Plant 11:62–74

Wiemken V (1994) Contributions of studies with *in vitro* culture systems to the understanding of the ectomycorrhizal symbiosis. In: Varma A, Hock B (eds) Mycorrhiza. Springer, Heidelberg, pp 411–425

Wilcox HE (1968) Morphological studies of the roots of red pine, *Pinus resinosa*. I. Growth characteristics and branching patterns. Am J Bot 55:247–254

Wong KKY, Fortin JA (1988) A Petri dish technique for the aseptic synthesis of ectomycorrhizae. Can J Bot 67:1713–1716

Woo HH, Hackett WP, Das A (1994) Differential expression of a chlorophyll A/B binding-protein gene and a proline-rich protein gene in juvenile and mature phase English ivy (*Hedera helix*). Physiol Plant 92:69–78

Zaczek JJ, Steiner KC, Neuser CW, Tzilkowski WM (2006) Effects of serial grafting, ontogeny, and genotype on rooting of *Quercus rubra* cuttings. Canadian Journal of Forest Research-Revue Canadienne de Recherche Forestière 36:123–131

Biotechnology and Cultivation of Desert Truffles

A. Morte(✉), M. Honrubia, and A. Gutiérrez

1 Introduction

Among the ascomyces truffles, there are several genera with an excellent record as edible fungi, and two of these are of considerable economic importance: *Terfezia* and *Tuber*. Of these two genera, only *Tuber* had been cultivated commercially (for decades), until now. More recently, biotechnological methods on fungal inoculum and mycorrhizal plant production, as well as plantation management, have been developed to cultivate, for the first time, some species of the *Terfezia* genus (Honrubia et al. 2001, 2005; Morte et al. 2004, 2006). These procedures are presented in this chapter. Here, we attempt to evaluate conclusions on the basis of recent truffle production data from the first field plantations.

The genus *Terfezia* belongs to the so-called "desert truffles" which are a complex family of mycorrhizal hypogeous fungi, mainly containing species of the genera *Terfezia, Picoa, Tirmania* and *Tuber*. Their geographical distribution is limited to arid and semiarid lands, mostly in countries around the Mediterranean basin, such as: southern Spain, Portugal, Italy, France, Hungary, Turkey, from Morocco to Egypt, Israel, the Arabian Peninsula, Iran, Iraq, Libya, Syria, and Kuwait. In addition, some desert truffle species have been found in South Africa (Botswana) (Marasas and Trappe 1973), in North America and Japan (Trappe and Sundberg 1977), and China (Wang, unpublished data).

Generally, the regions where desert truffles grow have an annual rainfall which ranges from 50 to 380 mm. The truffle season produces good yields if the rainfall ranges from 70 to 120 mm in North African countries and from 100 to 350 mm in countries of southern Europe. The rainfall distribution is very important as far as both quantity and the time of the rainfall are concerned; that is, no later than the beginning of December in North African and Middle Eastern countries and no later than the beginning of October in countries of southern Europe.

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Desert truffles are of considerable interest for ecological reasons because of the low water input required for cultivation, which makes them an alternative agricultural crop in arid and semiarid areas, and one of commercial interest because of the prices they fetch in the open market. Part of the mystique of truffles is, of course, their often extravagant cost. Desert truffles are cousins of the white fragrant Italian (*Tuber magnatum*) and Perigord (*Tuber nigrum*) truffles. However, the prices of the desert truffles are much lower than those of these *Tuber* species. One of the reasons is that desert truffles are not as strongly flavored as the *Tuber* species. It is probably a question of good marketing and presenting them in an attractive, glamorous way to people.

As desert truffles grow much more prolifically than *Tuber* species in general, they can be used in a much greater volume. Sizable quantities of several species of wild *Terfezia* are collected and marketed in southern Europe, parts of North Africa, and other countries bordering the Mediterranean. However, natural areas of desert truffles have been disappearing in the last 50 years. Large areas of the coastal desert in Egypt and Libya were mined in World War II. More recently, in Kuwait, certain aspects of the 1990–1991 Gulf War seem to have ruined many truffle-gathering areas. In Europe, this has been due to the huge building construction process taking place in these "sunny" areas over the last 5 years.

2 Chemical Composition

Desert truffles have been known to be edible for 3,000 years (Chang and Hayes 1978). Several studies on their chemical composition have shown that they are rich in proteins, amino acids, fiber, minerals and carbohydrates (Ackerman et al. 1975; Al-Delaimy 1977; Ahmed et al. 1981; Bokhary et al. 1987, 1989; Bokhary and Parvez 1993; Murcia et al. 2003).

The protein content, which averages 20% of the dry weight in desert truffles, is significantly higher than in most vegetables and other fungi. One 250-g serving of desert truffle can contribute 23–27% of the recommended daily intake of protein. These truffles could be cultivated in several of the developing countries of Africa and the Near East, making them important sources of protein for human consumption (Murcia et al. 2003). Also, the National Cancer Institute recommends that the daily diet contains between 25 g and 35 g of dietary fiber (Wu et al. 1994), values that are much higher than the average level actually consumed. Accordingly, one 250-g serving of truffles could contribute 16–22% of the recommended daily intake of fiber.

Our results on *Terfezia claveryi* (Fig. 1a, b) and *Picoa lefebvrei* showed good levels of fiber and monounsaturated fatty acids and few changes in the proximate composition of truffles during industrial processing (freezing and canning). As a consequence, the consumption of these processed truffles is recommended (Murcia et al. 2003).

One of the most interesting food properties of desert truffles is their antioxidant activity with regard to their ability to inhibit lipid oxidation (Murcia et al. 2002). *T. claveryi* and *P. lefebvrei* have higher percentages of oxidation inhibition than some common food antioxidants, like α-tocopherol, BHA (E-320), BHT (E-321) and propel

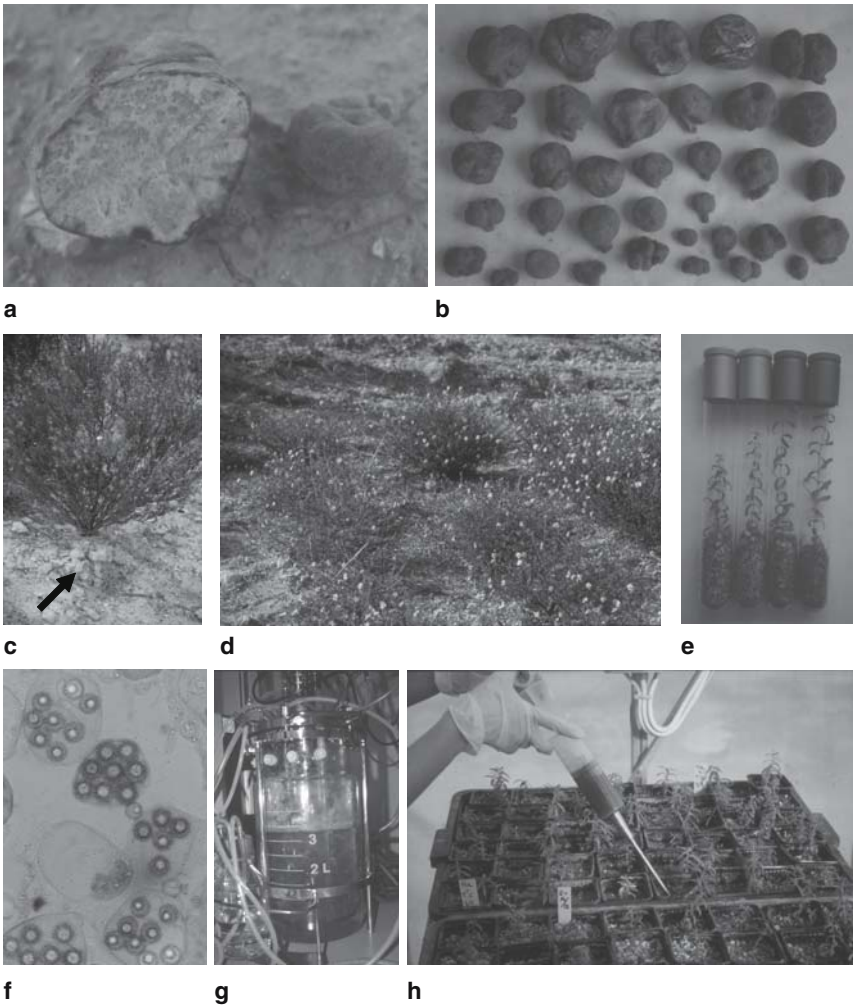

Fig. 1 **a,b** Ascocarps of *Terfezia claveryi*. **c** The host plant *Helianthemum almeriense* with a crack (*arrow*) in the nearby soil which indicates the presence of a truffle. **d** Plantation 1 of *H. almeriense* × *T. claveryi* in Lorca (Murcia, Spain). **e** In vitro mycorrhizal *H. almeriense* plants on vermiculite watered with the MH medium. **f** Asci and mature ascospores of *T. claveryi*. **g** Fermentor with a *T. olbiensis* mycelium culture. **h** In vivo inoculation of *H. almeriense* with a *T. claveryi* spore solution in the nursery

gallate (E-310), even after being subjected to the industrial freezing and canning processes (Murcia et al. 2002). More specifically, canned truffles present significant losses of antioxidant activity, while the frozen products suffer less extensive losses. Raw truffles exhibit a strong antioxidant activity as scavengers of several oxygen species. This finding supports the replacement of synthetic antioxidants with natural truffle extracts. Thus, raw and frozen truffles can be considered promising candidates for

industrial processing, thus permitting their year-round consumption. Furthermore, they can be considered as functional foods (Medina et al. 1999).

Despite their interesting chemical composition and antioxidant activity, a promising antibiotic activity has been detected in desert truffles (Rougieux 1963; Chellal and Lukasova 1995; Janakat et al. 2004, 2005). All these findings make desert truffles an interesting crop to be cultivated.

3 Biotechnological Aspects

Most desert truffles establish mycorrhizal symbiosis with species of the *Helianthemum* genus (relatives of the common rock rose cultivars) from the *Cistaceae* family. The fruiting bodies or truffles are often found nearby. They slightly push up the overlying soil, cracking it (Fig. 1c), and such cracks are used to help find the fungus. A screwdriver is generally used to push them up.

Their ecology changes depending on the host plant species. We can find the same truffle species, for example *Terfezia claveryi*, with the perennial species of *Helianthemum almeriense* (Fig. 1c) in an open low scrubland dominated by woody bushes. Alternatively, there are the same fungal species with *Helianthemum ledifolium*, annual, and *Helianthemum canariense*, perennial, in xerophytic meadows. The soil characteristics are also of a wide range with regard to texture (from sandy to calcareous), pH (acid and alkaline) and the rates values of electrical conductivity, organic carbon and the C/N rate.

The results presented in this chapter refer to three species of desert truffles: *T. claveryi*, *T. olbiensis* and *Picoa lefebvrei*. They are the most frequently found desert truffles in alkaline and calcareous soils from the Region of Murcia, in southeast Spain.

All these considerations should be taken into account in order to produce mycorrhizal plants suitable for different field conditions.

3.1 Plant Production

In order to establish mycorrhizal desert truffle plants in the field, it makes more sense to use perennial plant species than annual ones to maintain this culture for a minimum of 10 years. Accordingly, two perennial *Helianthemum* species have been selected for this purpose.

Helianthemum almeriense is a shrub that is 10–20 cm tall, whose distribution is restricted to southeast Spain and the north of Africa (Morocco). It appears in open places, in dry, stony, limestone, mica, marl, or marl with gypsum soils, even in sandy terrains, at an altitude of between 0 and 500 m (López-González 1993). *Helianthemum violaceum* has similar botanical characteristics but it colonizes a larger territory than *H. almeriense*, not only in Spain but also in southern Europe and North Africa.

The germination of their seeds is usually erratic. It is necessary to scarify the seeds for good germination results. Moreover, 50% of young seedlings normally die after 4–6 weeks in pots during nursery production. For these reasons, micropropagation of these plant species is an interesting option, not only as a variant of the traditional propagation method, but also to be able to control and better understand the nutritional requirements, thus enabling good mycorrhization (Morte and Honrubia 1997).

According to Morte and Honrubia (1992, 1997) and Zamora et al. (2006), micropropagation protocols mainly consist of:

1. Initial explants: shoot tips and nodal segments of in vitro germinated seeds.
2. Culture conditions: 22 ± 2°C, 40 µmol/m²/s Grow lux fluorescent light and 16-h photoperiod.
3. Culture medium: Murashige and Skoog (MS) (1962), with 0.8% Panreac-agar, 3% sucrose, pH 5.8.
4. Multiplication stage: MS medium with 0.46 µM kinetin (*H. almeriense*) or 0.023 µM kinetin (*H. violaceum*) for 4 weeks. The propagation system followed with these species was principally the formation of axillary buds and the culture of nodal segments. To a greater extent, the incorporation of kinetin to the medium caused the elongation of the explants, which allows them to be cut into several nodal segments. Kinetin was more effective than the other cytokinins tested (BA and 2iP) in the production of shoots. The multiplication rate was 7.72 shoots per initial explant for *H. almeriense* and 2.55 for *H. violaceum*.
5. Elongation stage: It was not necessary to change the culture medium for shoot elongation as the shoots elongated in the same multiplication medium. This was due to the propagation system used: the formation of nodal segments, which resulted in explants of up to 10 or 11 cm long at the multiplication stage. At the end of the multiplication stage on the other hand, the type of cytokinin used, and the relative low concentration, allowed the axillar-ramification formed shoots to reach an adequate length (2.5–3 cm) for their direct use at the following rooting stage. In *H. almeriense* and *H. violaceum*, therefore, the shoot multiplication rate is directly related to shoot elongation and the number of nodal explants available in each species.
6. Rooting stage: Spontaneous rooting was observed during the simultaneous multiplication and elongation stages in cultures with a low level of cytokinins, which varied between 25 and 80% for both plant species. This rooting percentage was improved to 100% with 1/4 macronutrient MS dilution for *H. almeriense*, and full MS for *H. violaceum*, and both without plant growth regulators for 3 weeks.
7. Weaning stage: The rooted plantlets were potted in a peat-sand-vermiculite mixture (3:1:1, v/v) and gradually exposed to reduced relative humidity for 2–3 weeks in the greenhouse. At the end of 1 month, approximately 95% of the plantlets survived.

In summary, the *in vitro* plant propagation of these *Helianthemum* species takes 10 weeks in total. It is a quick micropropagation protocol because plant multiplication, elongation and rooting may occur at the same subculture and, consequently, it is also a cheap protocol because of the small amount of plant growth regulators and manual labor required.

The *Helianthemum* plant production obtained directly from seed germination under nursery conditions takes at least 6 months to obtain plants suitable for fungal inoculation. This traditional propagation method is cheaper than in vitro propagation. However, both methods can be used for plant production, although it is advisable to use micropropagation if an in vitro culture laboratory is available.

3.2 Desert Truffle Inoculum Production

Two types of fungal inoculum have been used successfully to produce desert truffle mycorrhizal plants: spores and mycelium.

Spores from the *Terfezia* and *Picoa* species should be mature in order to facilitate their germination (Fig. 1f). The main problem found when using this type of inoculum is that the colleted ascocarps are immature for most of the time. We have observed that keeping the ascocarps covered by soil at 4°C for 10–15 days could induce spore maturation. The ascocarps with mature spores are then cut into pieces and dried at room temperature or in an oven at 60°C for 2 days. The dried ascocarp pieces are scratched and kept in glass jars at room temperature and in darkness until used. These spores are able to germinate even after two years in storage.

The spore suspension is performed taking into account the maturation of the spores. The spore suspension from well mature ascocarps consists of 10 g of scratched ascocarps per liter of distilled water. This spore solution is shaken overnight (12 h) before the inoculation of the plants. Each plant is inoculated with 5 ml of the spore solution, with a final spore concentration of 10^5–10^6 spores per plant. The spore germination is around 65% when the spores are from mature ascocarps.

The mycelium isolated from the desert truffles normally grows quite slowly, particularly during the first year in culture from the in vitro establishment. The best growth media are MMN (modified Melin-Norkrans) agar medium and PDA (potato dextrose agar) medium. pH should be adjusted at 7.0 if the ascocarps are from alkaline calcareous soils. Desert truffle mycelium can be used directly from the plates as inoculum for in vitro mycorrhizal synthesis (Morte et al. 1994; Morte and Honrubia 1995, 1997), and from liquid fermentation for both in vitro and in vivo inoculations.

Some ectomycorrhizal fungi can be grown on an industrial scale using liquid medium which is stirred and/or supplied with air within a large microbial culture vessel, called a bioreactor or fermentor (Fig. 1g). The fermentor culture has only been successful with the species *T. olbiensis* strain 111ET. The production of *T. olbiensis* mycelial inoculum was carried out in MMN liquid medium with a Braun BIOSTAT® B 5-l fermentor. A started inoculum is first prepared in a 250-ml flask with MMN liquid medium at pH 7.0 and grown for 20 days, then transferred to a 5-l fermentor for a 36-day period. During growth in the fermentor, the temperature is maintained at 23°C, the aeration rate is 1.5 l/min, the rotation speed is 100 *g*, with 60% dissolved oxygen. The pH of MMN medium is fixed at 7.0 and maintained during the fermentation process by adding 0.5 N NaOH when required. These conditions are set up at the beginning of the fermentation process and automatically

monitored by the bioreactor. Periodically, the stir speed is elevated to 1,000 *g* to break the mycelium and so to stimulate its growth.

During the fermentation process, *T. olbiensis* mycelium presents a 15-day acclimatization phase, in which there is no mycelium growth. After this, the mycelium grows in a sigmoid manner in parallel with a pH, and pO_2 decreases until reaching an exponential phase from day 29. At the end of the fermentation, the dry weight of mycelium is 1.16 g of mycelium per liter of medium.

These results show that the fungal growth in the bioreactor is much faster and more effective in terms of biomass production than the cultivation of fungi in flasks by traditional means (Le Tacon et al. 1985; Carrillo et al. 2004).

Finally, after 30 days of storage at 4°C, the fermentor-produced mycelium was used for mycorrhization trials and was able to infect *H. almeriense* plants as usual.

3.3 Mycorrhizal synthesis

The mycorrhizal synthesis between desert truffles and the *Helianthemum* species is carried out in different ways according to the type of fungal inoculum (spores or mycelium), the plant source (seedlings or micropropagated plantlets) and the culture conditions (in vivo or in vitro) used (Fig. 2).

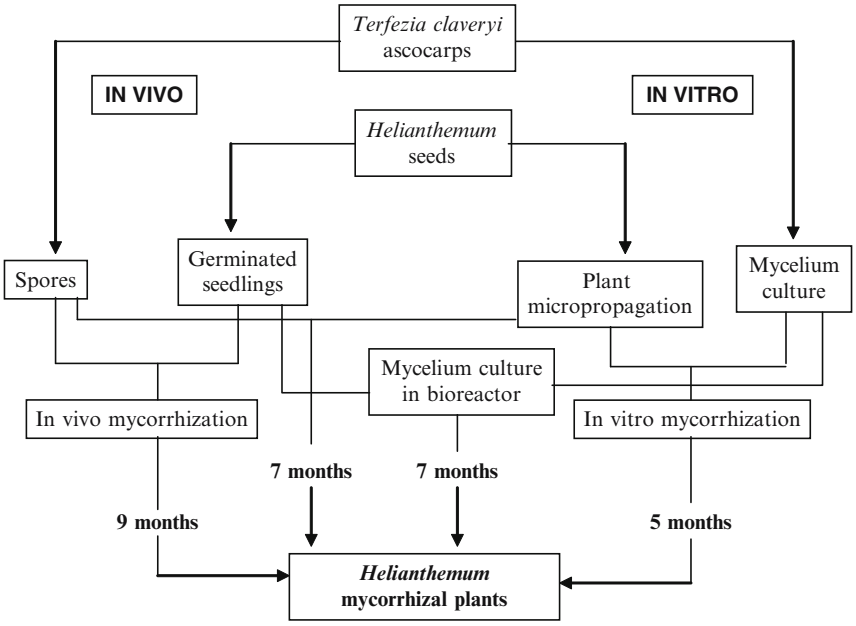

Fig. 2 In vivo and in vitro ways of producing desert truffle mycorrhizal plants and the time required for each of them

3.3.1 In vitro mycorrhization

In the in vitro system, when micropropagated *Helianthemum* plants and the pieces of agar with mycelium of *T. claveryi* or *P. lefebvrei* are used, is the quickest way (5 months in total) to produce mycorrhizal plants (Fig. 2). This synthesis is carried out on MH medium (Morte and Honrubia 1994, 1995), especially designed for this purpose, although it has been also obtained on MMN medium (Morte et al. 1994) both at pH 7.0 and solidified with agar. The rooted plantlets obtained in the MS medium are inoculated with two pieces of agar, 0.25 cm^2 in surface, with mycelium per tube. The tubes have a diameter of 2.5 cm and are 20 cm long. Twenty-five ml of medium are normally used per tube. The mycelium grows from the surface of the agar pieces, which serve as an inoculum, towards the interior of the culture medium in the test tube, within a 2-week period. This mycelial growth into the agar of the tube allows the mycelium to colonize the entire root system of the plantlets. The mycorrhization percentage obtained varies from 61 to 75% after 2 months in culture. This percentage is very similar for the synthesis obtained on both the MMN medium (Morte et al. 1994) and MH medium (Morte and Honrubia 1995). In contrast to MMN mycorrhization in the MH medium, however, there are no problems with the in vitro survival during the time that the rooted plantlets take to mycorrhize. This could be due to the fact that its composition is more similar to that of the MS micropropagation medium and has a greater number of nutrients than the MMN medium (Morte and Honrubia 1994).

Although the use of agar as a substrate for in vitro synthesis has given good mycorrhization percentages, sterilized vermiculite watered with MH liquid medium is strongly recommended for a semi-large-scale mycorrhizal plant production because it is more easily manageable than other types of substrate to transfer plants from in vitro to in vivo conditions (Morte et al. 2000; Gutiérrez 2001). Vermiculite also enables us to observe the growth of the mycelium on the root, and thus the start of mycorrhizal formation (Fig. 1e).

The mycorrhization percentages among the *Helianthemum* species (*H. almeriense, H. viscarium* and *H. violaceum*) and *T. claveryi* or *P. lefebvrei*, in in vitro conditions vary from 30 to 50% at 4 weeks, 60 to 75% at 8 weeks and 75 to 100% at 12 weeks from inoculation, assessed on cleared and stained root samples (Phillips and Hayman 1970), and estimated according to the gridline intersect method (Giovannetti and Mosse 1980). To optimise the best harvest time for these in vitro mycorrhizal plants, the amount of active mycelium in mycorrhizal roots has been estimated using the ergosterol measurement by HPLC at 280 nm, according to Gutiérrez et al. (2001). The ergosterol content in the mycorrhiza formed was analyzed at 4, 8 and 12 weeks after inoculation. The ergosterol present in the mycorrhizal roots of *H. viscarium* is less abundant than in the mycorrhizal roots in *H. almeriense*. In both the *Helianthemum* species, the ergosterol concentrations at 4 weeks after inoculation were 0.157 and 0.021 µg/mg of the fresh weight (FW) for *H. almeriense* and *H. viscarium*, respectively. These levels increase considerably at 8 weeks (0.214 and 0.163 µg/mg FW) and decrease markedly at 12 weeks (0.182

and 0.024 μg/mg FW). According to these data, the mycelium activity at 2 months is greater than that obtained at 3 months, even though mycorrhizal formation is slightly lower at 2 months than at 3 months. For this reason, we consider that 2 months is the optimum time to harvest in vitro mycorrhizal plants and transfer them to greenhouse conditions (Gutiérrez et al. 2001).

To transfer the in vitro mycorrhizal plants to ex vitro conditions, a mixture of sterilized soil, vermiculite 8:1 (v/v), is used. The soil is collected from areas where *Helianthemum* species grow naturally.

The advantages of this production system are that it is quick and nonseasonable because the mycelial inoculum and the micropropagated plants can be produced whenever required. However, an in vitro culture laboratory and specialized personnel are required which could elevate the final cost of the mycorrhizal plants.

3.3.2 In vivo Mycorrhization

Mycorrhizal plants are also obtained in nursery conditions directly from germinated seeds or from acclimatized micropropagated plants.

When seedlings are used, they must be 5–6 months old before fungal inoculation. Before this age, *Helianthemum* seedlings are too small and their root system is not properly developed and suitable for mycorrhization. These 6-months to year-old seedlings are inoculated with desert truffle spore suspension and it is necessary to wait 3 months to obtain good mycorrhizal plants. Spore suspension is directly applied close to the roots with a large needle connected to a syringe with the spore suspension (Fig. 1h). Nine months in total are required, this being the longest time spent to obtain our purpose (Fig. 2).

Two options exist to shorten this time: (1) the inoculation of acclimatized micropropagated plants with the spore suspension, and (2) the inoculation of seedlings with mycelial suspension obtained from fermentation in a bioreactor. In both cases, the time is reduced to 7 months. In the former case, the 2-months reduction is due to the quick plant micropropagation. When the micropropagated plants are acclimatized at 4 months, they have a good root system suitable for mycorrhization. However, 3 months are necessary for spores to colonize the *Helianthemum* roots well. In the second option, the saving in time is caused by the fast root colonization by the mycelium (only 1.5–2 months).

In short, the four options are designed to obtain a continuous desert truffle mycorrhizal plant production and to also avoid any limiting factor in this production.

3.4 Mycorrhiza Characterization

The characterization of the mycorrhiza formed in the *Helianthemum* root systems by these desert truffles is extremely important to assure high-quality produced

mycorrhizal plants. Furthermore, the maintenance of the desert truffle mycorrhiza should be checked in the root system after planting the mycorrhizal plants in the field, at least once a year. For this reason, the morphological and molecular characterization of the desert truffle mycorrhiza has been carried out.

3.4.1 Morphological Characterization

Under the stereomicroscope, four different mycorrhizal systems are observed in *H. almeriense* from different types of samples (naturally colonized roots, mycorrhiza formed in pots and in vitro-formed mycorrhiza): a club-shaped mycorrhiza, a capitate mycorrhiza, a moniliform mycorrhiza and a branched mycorriza (Gutiérrez et al. 2003). The most frequent type of these mycorrhizal systems, which appears in 46% of the studied plants, is the club-shaped mycorrhiza (Fig. 3a), an unbranched mycorrhiza, 6–8 µm in length. It has a whitish-ochre enlarged apex (0.6 µm) and a brownish-to-ochre constricted base of varying length. In general, external mycelium is observed around all the mycorrhizal systems. This mycelium is loose, bright white and not organized into a true sheath except in the in vitro mycorrhiza of both the fungal species used (*T. claveryi* and *P. lefebvrei*), where a true sheath is observed. The frequency of appearance of each mycorrhizal system neither changes with the different seasons nor with the mycorrhizal culture conditions of the fungal species used.

The four mycorrhizal systems present the same type of development at the cellular level, which only changes with the synthesis conditions but not with the fungal species. In field conditions, root colonization is mainly intracellular (Fig. 3b), forming an endomycorrhiza with large, septate and moniliform hyphae which surround the host nucleus that is frequently in a central position (Fig. 3b). Colonization only concerns the cortical cells of the roots. Intercellular hyphae are rarely observed. In no case is a sheath observed around the root; only in some cases do isolated hyphae appear around the roots (Gutiérrez et al. 2003). However, an ectomycorrhiza and ectendomycorrhiza without a sheath are formed in mycorrhiza synthesized with both desert truffles in pots under greenhouse conditions (Fig. 3c), similar to those described in *H. salicifolium* and *T. claveryi* mycorrhiza (Dexheimer et al. 1985). In the end, colonization is also intercellular in in vitro conditions, like a typical Hartig net, and only occasional hyphae are observed inside the cells (Fig. 3d). Groups of hyphae are frequently found between cortical cells, causing an important dilation of the intercellular spaces so that these cells show an elongated form. However, the main characteristic of the mycorrhiza formed *in vitro* is the presence of a well developed sheath, 50 µm thick (Fig. 3d). This sheath formation could be due to the vigorous mycelium growth on the MH mineral medium, which contains sucrose as a carbon source, and also result from the small physical space inside the test tubes which would force hyphae to surround the roots formed in vitro. Since the phosphorus content of the soil used for the mycorrhiza synthesized in pots was almost half (28.7 ppm) of that used for the in vitro synthesis in the MH medium (42.5 ppm), this may have influenced the type of mycorrhiza formed, although other factors varying between the experimental conditions could have contributed to this phenomenon

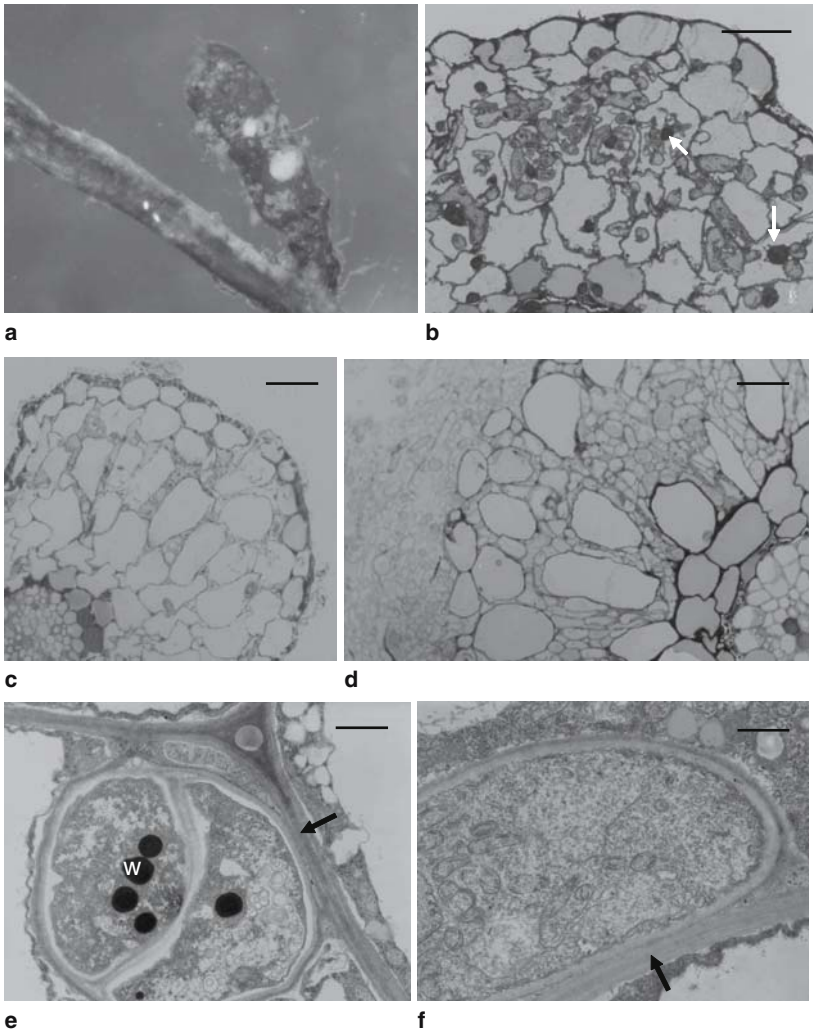

Fig. 3 **a** The club-shaped mycorrhiza in H. almeriense roots. **b** A mycorrhizal root of *H. almeriense* collected from field areas; colonization is intracellular. The nucleus of the host cell is in a central position surrounded by fungal hyphae (*arrows*). **c** Mycorrhizal roots of *H. almeriense* and *T. claveryi* in greenhouse conditions, forming an ectomycorrhiza and ectendomycorrhiza without a sheath. **d** Mycorrhizal root of *H. almeriense* synthesized under in vitro conditions with *T. claveryi* mycelium, with intercellular colonization and a sheath. **e,f** Intracellular hyphae with a host cell wall–fungal wall interface (*arrows*) and Woronin bodies (*w*) close to the septum. **b,c,d** *bars* 40 µm, **e** *bar* 0.35 µm, **f** *bar* 0.5 µm

(Gutiérrez et al. 2003). These data obtained for *H. almeriense* support those obtained for the *H. guttatum* and *Terfezia* and *Tirmania* species whose substrate fertility affects mycorrhiza morphology (Fortas and Chevalier 1992).

In conclusion, culture conditions can induce changes in mycorrhiza morphology and there is no clear barrier between the two main types of mycorrhiza organization in the *Helianthemum* species (Gutiérrez et al. 2003). Recently, two genes from *T. boudieri* (GenBank DV205797, DV205787) have been associated with ectomycorrhizas under pre-infection growth conditions, but with endomycorrhizas under mycorrhizal conditions. These genes could be involved in determining the type of association to be formed (Zaretsky et al. 2006).

Three ultrastructural studies on the mycorrhiza formed by *T. claveryi* with different *Helianthemum* species have confirmed the regular presence of *T. claveryi* intracellular hyphae in direct contact with the host plant wall (Fig. 3e, f), a localization which seems to be characteristic of the *T. claveryi* mycorrhiza organization (Dexheimer et al. 1985; Fortas 1990; Gutiérrez et al. 2003).

3.4.2 Molecular characterization

In order to identify different species of desert truffles inside the roots, DNA was extracted from 3–20 mg of ascocarps from different desert truffles species, their axenic mycelia and the mycorrhiza formed by *H. almeriense* x *T. claveryi*, according to the method described by Gutiérrez et al. (1995). The ITS region was successfully amplified by RFLP analysis. The ITS amplification product is a single band of 600 bp in size for all the isolates of *T. claveryi* as well as for the other studied species (*T. boudieri, T. olbiensis, T. arenaria* and *P. lefebvrei*) and for the mycorrhiza. It is possible to differentiate all these fungal species not only by a combination of the ITS region (without restriction), but also from the different patterns observed after treating the PCR fragments with the *Alu* I, *Hinf* I and *Msp* I restriction enzymes. Some of these ITS sequences of the rDNA from *T. claveryi, T. olbiensis* and *P. lefebvrei* are deposited in the GenBank.

The mycorrhiza ITS region restriction was performed with the enzymes *Alu* I and *Hinf* I. Restriction with *Alu* I gives two fragments of the same size than those obtained for *T. claveryi* (400 and 275 bp), and restriction with *Hinf* I gives two fragments of the same size than those also obtained for *T. claveryi* (300 bp).

4 Cultivation of the Desert Truffle *Terfezia claveryi*: Plantation Establishment and Management

An ecophysiological study on the mycorrhizal association *H. almeriense-T. claveryi* demonstrates that the water potential, transpiration, stomatal conductance and net photosynthesis are higher in mycorrhizal plants than in nonmycorrhizal plants. Moreover, this increase is higher in water-stressed plants than in well watered plants (Morte et al. 2000). Drought stress tolerance could be partly attributed to specific physiological mechanisms on chlorophyll content and gas exchange induced by the presence of *T. claveryi*. This means that the low water input required for cultivation makes them an alternative agricultural crop in arid and semiarid areas.

Furthermore, the introduction of desert truffles into dry environments may be a useful way to rehabilitate lands which have been considered unproductive to date. Both the productive nursery and agriculture sectors of nonirrigated land will be favored by the possibility of installing an alternative cultivation in what have been unproductive lands to date. They are products that will improve not only the quality of the land, avoiding soil erosion, but also the economy of social and economically depressed areas, and will add value to these territories.

Cultivation of desert truffles has been hampered by the difficulty of obtaining a good pure mycelium growth and good spore germination. Despite these difficulties, many mycorrhizal symbioses have been obtained between desert truffles and the *Helianthemum* species in greenhouses (Awameh et al. 1979; Cano et al. 1991; Chevalier et al. 1984; Gutiérrez 2001) and in in vitro conditions (Roth-Bejerano et al. 1990; Fortas and Chevalier 1992; Kagan-Zur et al. 1994; Morte et al. 1994). However, no plantations with mycorrhizal plants have been carried out to date.

Until the present time, eight desert truffle plantations have been organized in Spain (Table 1) using *H. almeriense* as a host plant mycorrhized with *T. claveryi*. These mycorrhizal plants were obtained in both nursery and *in vitro* conditions (see Section 3.3). Six of these plantations have been set up in the Region of Murcia, southeast Spain, at an altitude of 600 m. The first *T. claveryi* plantation was made up of 60 *H. almeriense* mycorrhizal plants in May 1999 at Zarzadilla de Totana (Lorca, Murcia, Spain) (Fig. 1d; Table 1). The last two new plantations were set up in spring 2006 in Andalusia,

Table 1 *Terfezia claveryi* production in the different plantations in Spain. (Data obtained up to September 2006)

Plantation	Place (Province)	No. of mycorrhizal plants	Plantation date	Management	Truffle production (kg/ha)		
					1999– 2004	2005	2006
1	Lorca (Murcia)	60	May 1999	Yes	1,147.6	0.6	1.6
2	Lorca (Murcia)	188	July 2001	No	4.8	-	-
3	Lorca (Murcia)	83	March 2002	No	19.5	0.02	-
4	Lorca (Murcia)	196	March 2002	Yes	29.4	600	120
5	Mula (Murcia)	80	March 2002	No	8.2	-	-
6	Lorca (Murcia)	640	January 2003	No	-	-	-
7	Sierra de María (Almería)	2,000	April 2006	In process	-	-	-
8	Chirivel (Almería)	3,000	April 2006	In process	-	-	-

specifically in the province of Almería, with 2,000 and 3,000 plants, respectively, as part of the Cussta Programme from the regional government of Andalusia.

In general, soils used for plantations are characterized by a clay-loamy texture, basic pH (8.5) and low rate values of electrical conductivity (123 µS/cm), organic carbon (0.9–3.9) and C/N rate (7–10). These characteristics, above all pH and texture, are different for other *Terfezia* species such as *T. arenaria* and *T. leptoderma* in Kuwait (Awameh and Alsheik 1978), Morocco (Khabar 1988) or Spain (Moreno et al. 1986), *T. pfeilli* in Botswana (Taylor et al. 1995) and *T. terfezioides* in Hungary (Bratek et al. 1996; Kovacs et al. 2003) which normally grow in acid and sandy soils.

The first cultivated desert truffles were obtained in April 2001, 23 months after the planting date, in plantation 1. Presently, this time has been reduced to 12 months with the adequate agricultural management.

We have developed a very simple management plantation protocol, after the experience acquired over the years, and after reducing the fructification time from the plantation from 23 months to 12 months (data obtained from plantations number 1 and 4). In short, it consists of mainly planting in spring, one single irrigation during the dry summer, at the end of August, of around 60–100 l/m^2, and a yearly weeding after the third year of plantation.

After testing in all seasons of the year, spring has been selected as the best time to set up a plantation owing to its moderate temperatures, the abundance of precipitation and the long photoperiod. The rainfall dependence of desert truffle fruiting is one of the most influential factors to be considered for a successful cultivation. In the region of Murcia, after years with a rainfall of between 350 and 400 mm, the estimated desert truffle production in natural areas varied between 50 and 170 kg Ha^{-1} (Honrubia et al. 2003). An irrigation system in the plantation is not necessary when the rainfall is available because the mycorrhizal association is well adapted to arid and semiarid climates (Morte et al. 2000). However, irrigation should be applied in dry years when the rainfall is less than 150 mm, at the end of the summer (August/September) and a second one at the beginning of the fruiting season (January/February) in very dry years. A drip irrigation system is recommended to save large amounts of water, although watering by sprinklers is also effective. The amount of water applied could range between 60 and 100 l/m^2 depending on the plant status. In the end, one manual yearly weeding is necessary, particularly after the third year of the plantation, in order to avoid plant competition for water and to maintain the open and sunny desert truffle ecosystem. A superficial weeding with a weeding hoe is recommended every 3 or 4 years in the same reason. To facilitate this management, a plantation frame of 3 × 3 or 3 × 2 m should be used.

The application of this plantation management is necessary to maintain the desert truffle production over time because without it, plantations have lost their productivity after 2 and 3 years (Table 1). Moreover, this management is essential during years of severe drought, like 2005 and 2006 in southeast Spain (Table 1). However, the desert truffle production fluctuates from one year to another in the same plantation, despite the management. These fluctuations could be due to other environmental or soil conditions, such as temperature and relative humidity, which always influence any crop production in the field.

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References

Ackerman LGJ, Vanwyk PJ, Du Plassis LM (1975) Some aspects of the composition of the Kalhari truffle of N'abba. South Afr Food Rev 2:145–147

Ahmed AA, Mohammed MA, Hami MA (1981) Libyan truffles "*Terfezia boudieri* Chatin". Chemical composition and toxicity. J Food Sci 11:145–147

Al-Delaimy KS (1977) Protein and amino acid composition of truffles. Can Inst Food Sci Technol J 10:221–222

Awameh M, Alsheikh A (1978) Laboratory and field study of four kinds of truffle (Kamah), *Terfezia* and *Tirmania* species for cultivation. Mushroom Sci X2:507–517

Awameh M, Alsheikh A, Ghawas S (1979) Mycorrhizal synthesis between *Helianthemum ledifolium, H. salicifolium* and four species of *Terfezia* and *Tirmania* using ascospores and mycelial cultures obtained from ascospores germination. Proceedings 4th North American Conference on Mycorrhizae. Fort Collins Colorado, USA

Bokhary HA, Parvez S (1993) Chemical composition of desert truffle *Terfezia claveryi*. J Food Comp Anal 6:285–293

Bokhary HA, Suleiman AAA, Basalah MO, Parvez S (1987) Chemical composition of desert truffles from Saudi Arabia. Can Inst Food Sci Technol J 20:336–341

Bokhary HA, Suleiman AAA, Basalah MO (1989) Fatty acid components of the desert truffle "Al-Kamah" of Saudi Arabia. J Food Protect 52:608

Bratek Z, Jakucs E, Boka K, Szedlay G (1996) Mycorrhizae between black locust (*Robinia pseudoacacia*) and *Terfezia terfezioides*. Mycorrhiza 6:271–274

Cano A, Honrubia M, Molina-Niñirola C (1991) Mycorrhizae in semi-arid ecosystems: synthesis of mycorrhizae between *Terfezia claveryi* Chat., *Picoa juniperi* Vit. and *Helianthemum almeriense* (Cistaceae). 3rd European Symposium on Mycorrhiza Sheffield, UK

Carrillo C, Díaz G, Honrubia M (2004) Improving the production of ectomycorrhizal fungus mycelium in a bioreactor by measuring the ergosterol content. Eng Life Sci 4:43–45

Chang ST, Hayes WA (1978) The biology and cultivation of edible mushrooms. Academic, New York

Chellal A, Lukasova E (1995) Evidence for antibiotics in the two Algerian truffles *Terfezia* and *Tirmania*. Pharmazie 50:228–229

Chevalier G, Dupré C, Riousset L, Dexheimer J (1984) Synthèse mycorhizienne entre *Terfezia leptoderma* Tul et diverses Cistacées. Agronomie 4:210–211

Dexheimer J, Gerardo, Leduc JP, Chevalier G (1985) Étude ultrastructurale comparée des associations symbiotiques mycorhiziennes *Helianthemum salicifolium – Terfezia claveryi* et *Helianthemum salicifolium – Terfezia leptoderma*. Can J Bot 63:582–591

Fortas Z (1990) Etude de trois espèces de Terfez: caractères culturaux et cytologie du mycelium isolé et associé à *Helianthemum guttatum*. Doctoral Thesis, Institut National de la Recherche Agronomique, Clermont-Ferrand

Fortas Z, Chevalier G (1992) Effects des conditions de culture sur la mycorrhization de l'*Helianthemum guttatum* par trois espèces de terfez des genres *Terfezia* et *Tirmania* d'Algerie. Can J Bot 70:2453–2460.

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol 84:489–499

Gutiérrez A (2001) Caracterización, micorrización y cultivo en campo de las trufas de desierto. Doctoral Thesis, Universidad de Murcia

Gutiérrez A, Honrubia M, Morte A, Díaz G (1995) Edible fungi adapted to arid and semiarid areas, molecular characterization and in vitro mycorrhization of micropropagated plantlets. Cah Options Mediterr 20:139–144

Gutiérrez A, Morte A, Honrubia M (2001) Ergosterol in desert truffle mycorrhizas. In: Crop and food research (ed) Edible mycorrhizal mushrooms and their cultivation. Christchurch, New Zealand, pp 1–4

Gutiérrez A, Morte A, Honrubia M (2003) Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorriza 13:299–307

Honrubia M, Gutiérrez A, Morte A (2001) Desert truffle plantations from South-East Spain. 3rd International Conference on Mycorrhiza, Adelaide, Australia.

Honrubia M, Morte A., Gutiérrez A, González F, Dieste C (2003) Las Turmas o Trufas de Desierto. In: Esteve-Selma MA, Lloréis-Pascual M, Martínez-Gallur C (eds) Los recursos naturales de la región de Murcia. Un Análisis Interdisciplinar. Servicio de Publicaciones de la Universidad de Murcia, Spain, pp 277–279

Honrubia M, Morte A, Gutiérrez A (2005) Six years of the *Terfezia claveryi* cultivation in Murcia (Spain). IV International Workshop on Edible Mycorrhizal Mushrooms, Murcia, Spain, p 70

Janakat SM, Al-Fakhiri SM, Sallal AKJ (2004) A promising peptide antibiotic from *Terfezia claveryi* aqueous extract against *Staphylococcus aureus in vitro*. Phytother Res 18:810–813

Janakat SM, Al-Fakhiri SM, Sallal AKJ (2005) Evolution of antibacterial activity of aqueous and methanolic extracts of the truffle *Terfezia claveryi* against *Pseudomonas aeruginosa*. Saudi Med J 26:952–955

Kagan-Zur V, Raveh E, Lischinsky S, Roth-Bejerano N (1994) Initial association between *Helianthemum* and *Terfezia* is enhanced by low iron in growth medium. New Phytol 127:567–570

Khabar L (1988) Le genre *Terfezia* Tull. (Terfass) de la Forêt de la Mamora (Région de Salé): etude systématique, ecologique, morphologique, cytologique et ultrastructurale. Université Mohammed V, Rabat

Kovacs GM, Vagvolgyi C, Oberwinkler F (2003) *In vitro* interaction of the truffle *Terfezia terfezioides* with *Robinia pseudoacacia* and *Helianthemum ovatum*. Folia Microbiol 48:360–378

Le Tacon F, Jung G, Mugnier J, Michelot P, Mauperin C (1985) Efficiency in a forest nursery of an ectomycorrhizal fungus inoculum produced in a fermenter and entrapped in a polymeric gels. Can J For Res 63:1664–1668

López-González G (1993) Plumbagináceas – Capparaceae. In: Castroviejo S, Aedo C, Cirujano S, Lainz M, Montserrat P, Morales R, Muñoz-Garmendia F, Navarro C, Paiva J, Soriano C (eds) Flora Ibérica. Plantas vasculares de la Península Ibérica e Islas Baleares, vol III. Serv Publ CSIC, Madrid

Marasas WFO, Trappe JM (1973) Notes on southern African Tuberales. Bothalia 11:139–141

Medina I, Satue-Gracia MT, German JB, Frankel EN (1999) Comparison of natural polyphenol antioxidants from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. J Agric Food Chem 47:4873–4879

Moreno G, Galán R, Ortega A (1986) Hypogeous fungi from continental Spain. Cryptogam Mycol. 7:201–229

Morte A, Honrubia M (1992) *In vitro* propagation of *Helianthemum almeriense* Pau (Cistaceae). Agronomie 12:807–809

Morte A, Honrubia M (1994) Método para la micorrización in vitro de plántulas micropropagadas de *Helianthemum* con micelio de *Terfezia claveryi*. Patent n° 9402430. Universidad de Murcia

Morte A, Honrubia, M (1995) Improvement of mycorrhizal synthesis between micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle). In: Elliot TJ (ed) Science and cultivation of edible fungi, vol 2. Balkema, Rotterdam, pp 863–868

Morte A, Honrubia M (1997) Micropropagation of *Helianthemum almeriense*. In: Bajaj YPS (ed), Biotechnology in agriculture and forestry. high-tech and mircropropagation, vol 40. Springer, Heidelberg, pp 163–177

Morte A, Cano A, Honrubia M, Torres P (1994) *In vitro* mycorrhization of micropropagataed *Helianthemun almeriense* plantlets with *Terfezia claveryi* (desert truffle). Agric Sci Finland 3:309–314

Morte A, Lovisolo C, Schubert A (2000) Effect of drought stress on growth and water relations of the mycorrhizal associations *Helianthemum almeriense-Terfezia claveryi*. Mycorrhiza 10:115–119

Morte A, Gutiérrez A, Honrubia M (2004) Desert truffle cultivation: a rehabilitation tool for degraded semiarid lands. In: Faz A, Ortíz R, García G (eds). IV International Conference on Land Degradation, Murcia, Spain

Morte A, Gutiérrez A, Bordallo JJ, Honrubia M (2006) Biotechnology and cultivation of desert truffles: an agricultural tool for degraded semiarid lands. 5th International Conference on Mycorrhiza, Granada, Spain.

Murcia MA, Matínez-Tomé M, Jiménez AM, Vera AM, Honrubia M, Parras P (2002) Antioxidant activity of edible fungi (truffles and mushrooms): losses during industrial processing. J Food Protect 65:22–30

Murcia MA, Martínez-Tomé M, Vera A, Morte A, Gutierrez A, Honrubia M, Jiménez AM (2003) Effect of industrial processing on desert truffles *Terfezia claveryi* and *Picoa juniperi* Vitt: proximate composition an fatty acids. J Sci Food Agric 83:535–541

Phillips IM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Roth-Bejerano N, Livne D, Kagan-Zur V (1990) *Helianthemum-Terfezia* relations in different growth media. New Phytol 114:235–238

Rougieux R (1963) Actions antibiotiques et stimulantes de la truffe du desert (*Terfezia boudieri* Chatin). Ann Inst Pasteur 105:315

Taylor FW, Thamage DM, Baker N, Roth-Bejerano N, Kagan-Zur V (1995) Notes on the Kalahari desert truffle, *Terfezia pfeilii*. Mycol Res 99:874–878

Trappe JM, Sundberg WJ (1977) *Terfezia gigantea* (Tuberales) in North America. Mycologia 69:433–437

Wu W, Williams WP, Kunkel ME, Acton JC, Wardlaw FB, Huang Y, Grimes LW (1994) Thermal effects on in vitro protein quality of red kidney bean. J Food Sci 59:1187–1191

Zamora M, Morte A, Gutiérrez A, Honrubia H (2006) *Helianthemum violaceum* Pers., a new host plant for mycorrhizal desert truffle plant production. 5th International Conference on Mycorrhiza, Granada, Spain

Zaretsky M, Kagan-Zur V, Mills D, Roth-Bejerano N (2006) Analysis of mycorrhizal associations formed by *Cistus incanus* transformed root clones with *Terfezia boudieri* isolates. Plant Cell Rep 25:62–70

The Fungal Transmitted Viruses

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1 Introduction

Most plant viruses are absolutely dependent on a vector for plant-to-plant spread. A number of different types of organisms work as vectors for different plant viruses. Plants, as sessile organisms, cannot transmit viruses except for some instances of seed or pollen transmission and the movement of plants resulting from human intervention. Thus, the great majority of plant viruses are dependent for their spread upon efficient transmission from plant to plant by specific vectors.

Vector transmission is a specific event in the virus life cycle. Virus-encoded determinants specifically interact with the vector, thereby facilitating virus transmission, and various plant viruses utilize different, but specific, vectors to facilitate their spread. Different organisms such as insects, fungi, nematodes, animals and arthropods are recognized as vectors for various plant viruses, but in most cases, viruses of a given taxon have a specific type of vector (e.g., potyviruses are aphid-transmitted). These observations suggest that virus particles as well as vectors have specific sites that mediate their recognition. The coat protein (CP) of a plant virus has been shown to play an important role in transmission, and particular amino acids within the CP have been shown to be essential for this process (Brown et al. 1995; Campbell 1996; Gray 1996; Gray et al. 1999; Pirone and Blanc 1996). Recent work with *Cucumber necrosis virus* (CNV) has suggested that attachment of virions to vector zoospore is an important aspect of the transmission process.

The concept of soilborne vectors came into existence in 1958 with the study of the nematode, *Xiphinema index*, vector of *Grapevine fanleaf virus* (Hewitt et al. 1958) and with the association of Chytrid fungus, *Olpidium brassicae*, with transmission of bigvein disease of lettuce (Fry 1958; Grogan et al. 1958). Soilborne transmission occurs by two different organisms: fungi and nematodes.

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2 Fungi Transmitting Plant Viruses

Thirty soilborne viruses or virus-like agents are transmitted by five species of fungal vectors. Two species of Chytridiomycetes (*O. brassicae* and *O. bornovanus*) (Table 1) and three species of Plasmodiophoromycetes (*Polymyxa graminis, P. betae*, and *Spongospora subterranea*) (Table 2) are recognized as vectors of plant viruses. All five species are obligate parasites of plant roots and have similar developmental stages. Plasmodiophorids are now classified as protists rather than true fungi. Plasmodiophorids are currently classified in the order Plasmodiophorales and in the family Plasmodiophoraceae, wherein *Polymyxa* and *Spongospora* represent two of ten genera (Dick 2001). They survive from crop to crop as resting spores that produce zoospores and infect the host. The plasmodiophorids have bi-flagellate, heterokont zoospores and form cystosori consisting of single-celled resting spores formed by division of the thallus (Tomlinson 1958; Barr 1979). Plasmodiophore-transmitted viruses are mostly positive strand RNA viruses.

The chytrids are identified by the size of the posteriorly-uniflagellate zoospores, which have a characteristic "jerky" swimming pattern, and by the morphology of their single-celled resting spores (Campbell and Sim 1994; Lange and Insunza 1977).

Currently, two types of virus–fungal vector relationship are recognized. They are characterized by the method of virus acquisition and by the location of virions relative to the resting spore. The in vitro transmission method involves in vitro acquisition, in which virions in solution are adsorbed to the surface of zoospores,

Table 1 Viruses transmitted by *Olpidium* species (Rochon et al. 2004)

Virus	Acronym	Genus	Fungal vector	Acquisition mode
Tombusviridae				
Cucumber necrosis virus	CNV	*Tombusvirus*	*O. bornovanus*	In vitro
Cucumber leaf spot virus	CLSV	*Aureusvirus*	*O. bornovanus*	In vitro
Cucumber soil borne virus	CSBV	*Carmovirus*	*O. bornovanus*	In vitro
Melon necrotic spot virus	MNSV	*Carmovirus*	*O. bornovanus*	In vitro
Squash necrosis virus	SqNV	*Carmovirus*	*O. bornovanus*	In vitro
Red clover necrotic mosaic virus	RCNMV	*Dianthovirus*	*O. bornovanus*	In vitro
Chenopodium necrosis virus	ChNV	*Necrovirus*	*O. brassicae*	In vitro
Lisianthus necrosis virus	LNV	*Necrovirus*	*O. brassicae*	In vitro
Tobacco necrosis virus-A	TNV-A	*Necrovirus*	*O. brassicae*	In vitro
Tobacco necrosis virus-D	TNV-D	*Necrovirus*	*O. brassicae*	In vitro
Viruses in unassigned family				
Mirafiori lettuce virus	MiLV	*Ophiovirus*	*O. brassicae*	In vivo
Tulip mild mottle mosaic virus	TMMMV	*Ophiovirus*	*O. brassicae*	In vivo
Freesia leaf necrosis virus	FLNV	*Varicosavirus*	*O. brassicae*	In vivo
Lettuce big vein virus	LBVV	*Varicosavirus*	*O. brassicae*	In vivo
Lettuce ring necrosis virus	LRNV	*Varicosavirus*	*O. brassicae*	In vivo
Tobacco stunt virus	TSV	*Varicosavirus*	*O. brassicae*	In vivo

Table 2 Viruses transmitted by Plasmodiophoroid fungal vectors (Rochon D. et al. 2004)

Virus	Acronym	Genus	Fungal vector	Acquisition mode
Potyviridae				
Barley mild mosaic virus	BaMMV	*Bymovirus*	*P. graminis*	In vivo
Barley yellow mosaic virus	BaYMV	*Bymovirus*	*P. graminis*	In vivo
Oat Mosaic virus	OMV	*Bymovirus*	*P. graminis*	In vivo
Rice necrosis Mosaic virus	RNMV	*Bymovirus*	*P. graminis*	In vivo
Wheat spindle streak mosaic virus	WSSMV	*Bymovirus*	*P. graminis*	In vivo
Wheat yellow mosaic virus	WYMV	*Bymovirus*	*P. graminis*	In vivo
Viruses in unassigned family				
Beet necrotic yellow vein virus	BNYVV	*Benyvirus*	*P. betae*	In vivo
Beet soilborne mosaic virus	BSMV	*Benyvirus*	*P. betae*	In vivo
Indian peanut clump virus	IPCV	*Pecluvirus*	*P.graminis*	In vivo
Peanut clump virus	PCV	*Pecluvirus*	*P.graminis*	In vivo
Chinese wheat mosaic virus	CWMV	*Furovirus*	*P.graminis*	In vivo
Oat golden stripe virus	OGSV	*Furovirus*	*P.graminis*	In vivo
Rice stripe necrosis virus	RSNV	*Furovirus*	*P.graminis*	In vivo
Soilborne cereal mosaic virus	SBCMV	*Furovirus*	*P.graminis*	In vivo
Soilborne wheat mosaic virus	SBWMV	*Furovirus*	*P.graminis*	In vivo
Sorghum chlorotic spot virus	SrCSV	*Furovirus*	*P.graminis*	In vivo
Potato mop top virus	PMTV	*Pomovirus*	*S. subterranea*	In vivo
Beet soilborne virus	BSBV	*Pomovirus*	*P.betae*	In vivo
Beet virus Q	BQ	*Pomovirus*	*P.betae*	In vivo
Unclassified virus				
Aubian wheat mosaic virus	AWMV	*?*	*P.graminis*	In vivo
Watercress yellow spot virus	WYSV	*Tombusvirus*	*S. subterranean*	?

but the virions are not located within the resting spores. The in vivo transmission method involves in vivo acquisition, in which virus enters the thallus as it grows in a virus-infected host, and the virus is located within resting spores.

2.1 Viruses Transmitted by Olpidium Species

2.1.1 Tombusviridae

Thirteen viruses are presently recognized as members of the genus *Tombusvirus*. CNV, a member of the genus *Tombusvirus*, is a 30-nm spherical virus with a monopartite positive-sense RNA genome (Rochon and Tremaine 1989). CNV is naturally transmitted in the soil by zoospores of the fungal vector *Olpidium bornovanus*. Virus is adsorbed onto the plasma membrane of zoospores and then enters into roots upon zoospore encystment. (Kakkani et al. 2001). Various studies have shown that CNV CP contains determinants for its interaction with zoospores of *O. bornovanus* (McLean et al. 1994; Rochon and Tremaine 1989). A single amino acid mutation (Glu to Lys) in

the CNV CP shell domain results in lowered transmission efficiency of CNV by *O. bornovanus*. In vitro binding studies demonstrated that this mutant bound zoospores less efficiently than CNV, indicating that specific regions of the CNV coat protein can mediate zoospore adsorption (McLean et al. 1994). CNV was mechanically passaged 12 times through *Nicotiana clevelandii*, and individual local lesions were isolated following inoculation of cucumber cotyledons. The CP ORFs and flanking regions of six putative transmission mutants determined by reduced transmissibility were amplified by RT-PCR and cloned in place of the WT CNV CP ORF in an infectious CNV cDNA clone. The cloned region was then sequenced to determine the presence of mutations. Transcripts of each of the clones were inoculated onto plants, and purified virus from infected plants was tested for transmissibility. Of 87 local lesions analyzed, 7 were ultimately found to contain virus with reduced transmission.

Results of the transmission tests show that cloned mutants designated LLK8, LLK10, LLK63, LLK82, LLK84, and LLK85 were less transmissible than WT CNV (transmission efficiency, 96%). LLK8, LLK10, and LLK63 transmitted at lower efficiencies (i.e., 21, 27, and 14%, respectively), whereas LLK82, LLK84, and LLK85 transmitted at higher efficiencies (75, 50, and 76%, respectively). An uncloned mutant (LLK26) also transmitted with reduced efficiency (10%). Sequence analysis of LLK26 showed that it is identical to LLK8. (Kakkani et al. 2001). It was found in studies that these mutants are due to affected amino acids locating in the shell or protruding domain.

2.1.1.1 Molecular Structure

The structure of the CNV, *Cucumber leaf spot virus* (CLSV), *Red clover necrotic mosaic virus* (RCNMV), and the carmovirus capsids is based on studies of the X-ray crystal structures of *Tomato bushy stunt virus* (TBSV, tombusvirus) and *Turnip crinkle virus* (TCV, carmovirus) particles (Harrison et al. 1978; Hogle et al. 1986) as shown in Fig. 1. The structures of the CNV CP subunit and particle have been obtained using homology modeling. The CP subunit folds into three distinct domains: the RNA binding domain (R) located in the interior of the particle; the shell domain (S), which forms the particle backbone; and the protruding (P) domain, which projects outward from the particle. The R and S domains are connected by the *arm* and the S and P domains are connected by a short hinge (h). The CP subunit can adopt three different conformational arrangements, termed A, B, and C. Three ordered C subunit arms are interconnected at the particle threefold axis to form an internal network called the β-annulus that stabilizes the particle. The remaining A and B subunit arms remain disordered in the particle interior. The CP P domains from adjacent C/C or A/B subunits form dimers giving rise to 90 projections on the particle surface. The necrovirus CP subunit folds in a similar fashion except that it lacks the P domain and thus the 90 clusters on the particle surface (Oda et al. 2000).

A conserved polymerase encoding ORF is located near the 5′ end of the genome which can be interrupted either by a stop codon or a −1 frameshift that is occasionally read through to produce the polymerase encoding portion of the protein. All of the

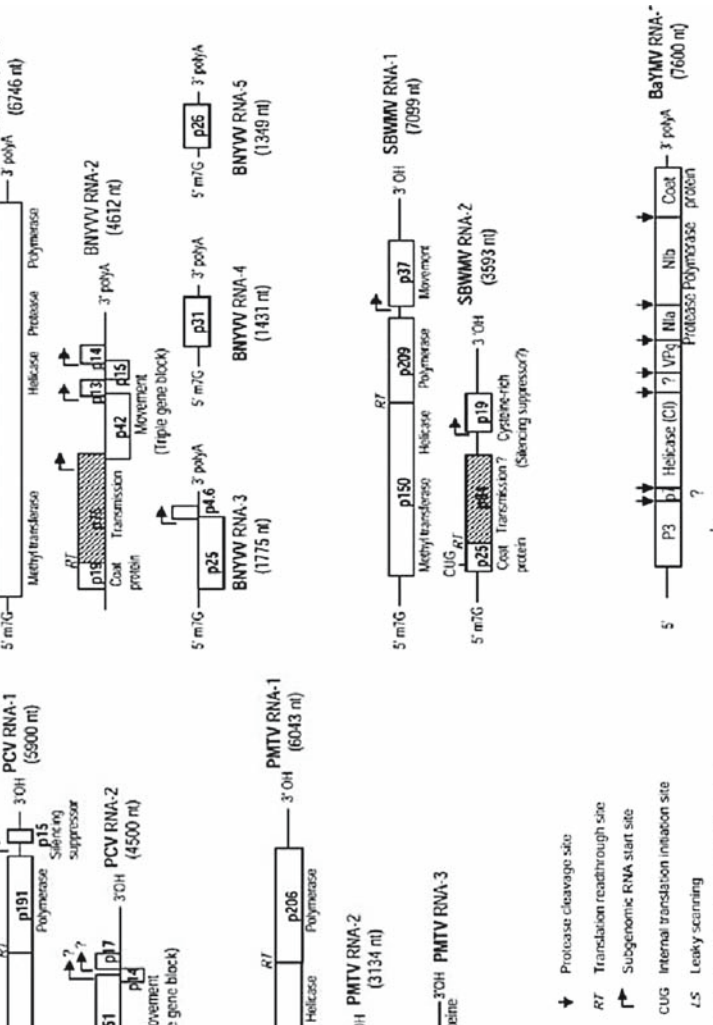

Fig. 1 Genome structures of viruses transmitted by plasmodiophorid vectors. *Hatched boxes* highlight the coat protein ORF believed to be responsible for transmission specificity. Main functions of proteins are shown as well as expression strategies

viruses in *Tombusviridae* lack the helicase domain often found upstream of the polymerase domain in viral replicases. The movement proteins (MP) of CNV and CLSV form one class (Reade et al. 1999; Miller et al. 1997); RCNMV another and the necroviruses a third (Lomell et al. 2000). The MP of RCNMV, although it may be in a different class, can assist both CNV and CLSV in local and systemic movement (Reade et al. 2000, 2003). CNV p20 is a potent suppressor of gene silencing (D.M.R. and Y. Xiang, unpublished observations). There is limited similarity between CNVp20 and CLSV p17, suggesting that p17 may also be a silencing suppressor (Reade et al. 1999, 2003), although this requires confirmation.

2.1.1.2 In Vitro Transmission

In vitro transmission is found with two species of *Olpidium* and with the polyhedral viruses that are all in the Tombusviridae family except *Satellite tobacco necrosis virus* (STNV) (Murphy et al. 1995). This transmission mechanism was first proposed when the TNV–*O. brassicae* interaction was clarified and compared to that already established for Lettuce big-vein virus (LBVV)–*O. brassicae* (Campbell and Fry 1966).

In vitro acquisition begins when virus-free zoospores released either from resting spores or from vegetative sporangia encounter virions in water. Virions are tightly and specifically adsorbed to the zoospore membranes (Stobbs et al. 1982; Temmink et al. 1970; Campbell 1996). Adsorption probably involves receptors in the zoospore membrane and the CP of the virions. Only virions encapsidated with the CNV CP were transmitted by the vector. The protruding domains of CNV CP may specifically interact with receptors on the zoospore plasma membrane. On the other hand, there are no protruding domains on the virions of TNV-A or TNV-D, which are transmitted by *O. brassicae* (Coutts et al. 1991; Meulewaeter et al. 1990). Either a protruding domain per se is not required or the recognition mechanism differs between *Olpidium* species. (Campbell et al. 1995).

In vitro acquisition is unique among plant viruses in that it occurs outside living cells; thus, the epidemiology of these viruses is unusual. Virus in the soil originates from infected roots during the crop season but from any infected host tissue after the crop is harvested.

2.2 Necrovirus

Two new members of necrovirus groups are *Chenopodium necrosis virus* (ChNV), isolated from river water (Tomlinson et al. 1983), and *Lisianthus necrosis virus* (LNV) (Iwaki et al. 1987). These were acquired in vitro by *O. brassicae*. The necrovirus CP subunit folds in a similar fashion as CNV but it lacks the P domain and thus the 90 clusters on the particle surface (Oda et al. 2000). Several modes of transmission have been reported in necroviruses: fungus, beetle, seed, soil,

mechanical, and vegetative propagation of hosts. *Melon necrotic spot virus* (MNSV) has been reported to have both a fungal and a beetle vector (Campbell 1996).

2.3 Carmovirus

Cucumber soilborne virus (CSBV) is transmitted by the squash strain of *O. bornovanus* but not by the cucumber strain. The complete sequence of the nucleotides in carmovirus has been established (Guilley et al. 1985) and shown to encode four ORFs. There is a 69-nucleotide 5′ leader sequence before the first AUG. There is a UAG termination codon that would give a protein of 28 kDa, and a UAA codon that would give a protein of 86 kDa. Both proteins are found in in vivo and in vitro.

Besides genomic RNA, two sgRNA species of 1.7 and 1.45 kb are produced in vivo and encapsulated in virus particles (Carrington and Morris 1984). They are 3′ co- terminal with the genomic RNA and are the mRNAs for ORFs 2 and 3 and for ORFs 4, respectively.

2.4 Dianthovirus

This genus with three viruses is categorized as NVST (nonvector soil transmission) and is included in the Tombusviridae A fungal vector may be either *O. brassicae* (Gerhardson and Insunza 1979); or *O. bornovanus* (Lange and Insunza 1977; Macfarlane 1982). Dainthoviruses differ from other members of Tombusviridae in that their genome is divided between two RNA species, RNA1 and RNA2.

2.5 Viruses of Unassigned Family

Unassigned family of viruses contains either rod shaped or filamentous particles and belong to one of the two genera, the *Ophioviruses* or the *Varicosaviruses*. Mirafiori lettuce virus (MiLV) (Lot et al. 2002; Roggero et al. 2000; Van der Wilk et al. 2002) and the serologically related *Tulip mild mottle mosaic virus* (TMMMV) (Morikawa et al. 1995; Roggero et al. 2000) are both ophioviruses, whereas the remaining are varicosaviruses (Mayo 2000). Ophiovirus-like particles have been observed in plants displaying symptoms of freesia leaf necrosis (Milne 2002), which suggests, as in the case of lettuce big-vein disease, that both an ophiovirus and a varicosavirus are present in diseased plants. Similarly, an ophiovirus distinct from MiLV has been found in association with plants infected with lettuce ring

necrosis (Torok and Vetten 2002; Vaira et al. 2003; Vetten et al. 1987), again suggesting dual infection.

2.5.1 Molecular and Genomic Structure of Ophiovirus

Ophiovirus particles are naked, finely filamentous structures of about 3 nm that form kinked circles of at least two different contour lengths (Roggero et al. 2000; Vaira et al. 2003). The virions are unstable, present at low concentrations in infected tissue and stain faintly, making their identification difficult. In the case of MiLV, it has been shown that virions encapsidate both the minus- and positive-sense RNAs.

The complete genome sequence of MiLV has recently been determined (Van der Wilk et al. 2002). The genome consists of four RNA components with sizes of approximately 7.8, 1.8, 1.5, and 1.4 kb. Each RNA is encapsidated in both polarities with approximately equal amounts of each polarity. The negative strand of RNA-1 contains two ORFs: one for a protein with a predicted molecular weight of 25 kDa (p25) and the other for a protein of 263 kDa (p263). The negative strand of RNA-2 has an ORF for a putative 55-kDa protein (p55) and the positive strand for a putative 10-kDa protein (p10). Thus RNA-2 may be ambisense but this requires experimental confirmation. RNA-3 encodes the CP (48.5 kDa) from the negative-sense strand. The RNA-4 negative-sense RNA can encode two proteins of 37 kDa (p37) and 10.6 kDa (p10.6), with the possibility that the 10.6 kDa protein could arise by a +1 translational frame shift of p37. p263 shares a high degree of sequence similarity with the corresponding protein of other sequenced ophioviruses (Naum-Ongania et al. 2003; Van der Wilk et al. 2002; Vaira et al. 2003). Vaira et al. (2003) designed primers for an ophiovirus-specific RT-PCR test based on sequence similarities in conserved portions of the ophiovirus RNA-1 polymerase encoding region. Phylogenetic studies based on comparisons of amino acid sequences of the conserved portions of the polymerase of MiLV, as well as two other ophioviruses, *Ranunculus white mottle virus* (RWMV) and *Citrus psorosis virus* (CPsV), suggest that ophioviruses represent a monophyletic lineage within the negative-strand RNA viruses, but one that is distinct from both the segmented and nonsegmented negative-strand viruses.

2.5.2 Molecular and Genomic Structure of Varicosavirus

Varicosavirus particles are rod-shaped with modal length of 320–360 nm and with a diameter of 18 nm. Virions do not contain a lipid envelope, are unstable, and consist of a single coat protein of about 45–48 kDa. LBVV a type member of Varicosavirus genus is the most extensively characterized (Kuwata et al. 1983; Mayo 2000). The varicosavirus genome is composed of two single-stranded RNAs (RNA-1 and RNA-2). Virions encapsidate both the positive- and negative-sense genomic RNAs (Sasaya et al. 2001), thus accounting for the presence of dsRNA in purified virion RNA preparations. The complete nucleotide sequence of LBVV

RNA-1 and a partial sequence of RNA-2 have been determined. RNA-1 is approximately 6.8 kb and is predicted to encode a protein of 232 kDa (p232) from the negative-sense strand. p232 contains the conserved functional motifs for the viral replicase. LBVV RNA-2 is about 6.5 kb and contains the CP ORF (p44.5) on the negative-sense strand near the 5′ terminus. The remaining sequence of LBVV RNA-2 has not been reported. Comparisons of amino acid sequences of LBVV p232 indicate that this protein is most similar to plant rhabdovirus L protein polymerases. Similarly, comparisons of the LBVV CP indicate a close relationship to rhabdovirus nucleoproteins (Sasaya et al. 2001, 2002).

2.5.3 In vivo Transmission

In in vivo acquisition, virus is believed to be within zoospores when they germinate from resting spores or emerge from sporangia. As with in vitro acquisition, the means by which virus enters the root cell cytoplasm following injection of the protoplast is not known. Several viruses are included in this category, and the model is based on vector virus combinations that have been tested thoroughly for *O. brassicae*–LBVV (Campbell 1962; Campbell and Grogan 1963, 1964; Tomlinson and Garrett 1964), *P. graminis–Soilborne wheat mosaic virus* (SBWMV) and *Barley mild mosaic virus* (BaMMV) (Rao and Brakke 1969; Adams et al. 1988). Virus acquisition occurs as the fungus develops within a virus-infected host cell, even within the first vegetative generation of the vector (Campbell and Grogan 1964). The process by which virions are acquired and transmitted is unknown.

3 Viruses Transmitted by Plasmodiophorids

Currently about 20 viruses are known to be transmitted by the plasmodiophorid vectors. The viruses belong either to the genus *Bymovirus* in the family Potyviridae or to one of four genera (the *Beny-*, *Furo-*, *Peclu-*, or *Pomoviruses*) in an unassigned family (Table 2). With the exception of *Watercress yellow spot virus* (WYSV), which has icosahedral particles (Clay and Walsh 1996), viruses transmitted by plasmodiophorids are nonenveloped, rod-shaped, or filamentous virions composed of a single repeating coat protein subunit and multipartite, positive-polarity, ssRNA genomes (Van et al. 2000). *Polymyxa graminis* transmits all of the bymo-, furo-, and pecluviruses as well as the benyvirus, *Rice stripe necrosis virus* (RSNV). *P. betae* transmits the remaining benyviruses and two pomoviruses, *Beet soil-borne virus* (BSBV) and *Beet virus Q* (BVQ), whereas *S. subterranea* transmits *Potato mop-top virus* (PMTV) and the unclassified WYSV. It is believed that all plasmodiophorid transmitted viruses are acquired in vivo (Table 2).

Host-specificity exists in the plasmodiophorid vectors. *S. subterranea* f. sp. *nasturtii* is specific for watercress (Tomlinson 1958). Host-specialization was the primary basis for describing *P. betae* as a species distinct from *P. graminis* (Barr

1979; Keskin 1964). Although the two species are morphologically and karyotypically (Braselton 1988) identical, the first molecular genetic analysis supports their distinction (Mutasa et al. 1993). Also, RFLP patterns of ribosomal DNAs clearly distinguished *P. betae* from *P. graminis*, and even differentiated 13 isolates of *P. graminis* into two groups (Ward et al. 1994).

Adams et al. (2001) showed that the readthrough domains of beny-, furo-, and pomoviruses as well as the P2 proteins of bymoviruses each contain two complementary transmembrane domains (T1 and T2), the second of which is deleted in viruses that have lost the ability to be transmitted. The region between T1 and T2 is predicted to be on the inside of the membrane and, therefore, the virus would initially be on the outside. The model predicts that the conserved transmembrane regions would assist virus in moving from the host cell cytoplasm across the plasmodial membrane (Adams et al. 1988; Kanyuka et al. 2003).

3.1 Development cycle of Plasmodiophorids

The zoospore encysts on a host root and then germinates to form a prepenetration swelling, i.e., adhesorium. From this, the protoplast is "injected" into the host cell by a bullet-like mechanism. The protoplast then grows into a multinucleate plasmodium, which eventually converts into a sporangium to release further zoospores. As an alternative, the multinucleate plasmodium can convert into numerous thick-walled resting spores, which are released when the host cells decay and can survive for many years in soil. They germinate eventually to release zoospores.

3.2 Transmission

Adams et al. (2001) suggested that in the case of viruses transmitted by plasmodiophorid vectors, transmembrane regions present in the coat protein readthrough could play a role in facilitating particle movement across the membrane. Direct evidence for the presence of virus in zoospores and sporangia has been obtained in the case of *Beet necrotic yellow vein virus* (BNYVV) and BaMMV (Chen et al. 1991; Dubois et al. 1994; Rysanek et al. 1992). In plasmodiophorid-transmitted viruses, this might occur with the aid of the two CP transmembrane regions (Adams et al. 2000). Unlike the transmission-related proteins of the beny-, furo-, and pomoviruses that are covalently associated with virus particles by virtue of being a readthrough product of CP, the pecluvirus p39 putative transmission protein and the bymovirus P2 transmission protein are expressed independently from CP. This raises the question as to how these proteins might facilitate transmission of virus particles.

3.3 General Genome Structure of Viruses Transmitted by Plasmodiophoroids

Figure 2 shows the genome structures of BNYVV, SBWMV, *Peanut clump virus* (PCV), PMTV, or *Barley yellow mosaic virus* (BaYMV). Some common elements are present among the viruses. One is the possession of a readthrough mechanism inSBWMV, PCV, and PMTV (Diao et al. 1999). Another is that the terminator codon for CPs of beny-, furo-, and pomoviruses can be readthrough, producing a CP with a carboxyl-terminal extension. Three distinctive movement strategies may be employed by plasmodiophorid-transmitted viruses. Recognition of these differences may be relevant to the transmission process. It has recently been reported that the SBWMV movement protein can be found within the resting spores and sporangia of *P. graminis* (Driskel and Verchot-Lubicz 2003). This raises the possibility that the specific movement function of SBWMV may contribute to its ability to be transmitted.

3.3.1 Furoviruses

The furoviruses form three vector-host groups: those infecting grasses and transmitted by *P. graminis*; those infecting chenopodiaceous plants and transmitted by *P. betae*; and the monotypic PMTV transmitted by *S. subterranea* f. sp. *subterranea*. The inclusion of PCV and *Indian peanut clump virus* (IPCV) in the grass group is justified on the basis of virus–host–vector relationships. Both viruses are transmitted by *P. graminis*, but peanut is a noncompatible host in which the vector cannot acquire virus, whereas *Sorghum* spp. are the normal, compatible hosts of virus and vector (Thouvenel and Fauquet 1981). Like BNYVV (Kruse et al. 1994), members of the furoviruses (including the tentative ones) have rod-shaped virions and 2–5 ssRNA components that are numbered in order from RNA-1, the longest RNA (Murphy et al. 1995). The diversity of genomic arrangements means that the tentative furoviruses should be separated into subgroups (Kashiwazaki et al. 1995; Shirako and Wilson 1993). Virions are not enveloped. Virions are more than one type of particle; differ in size, but within the same range. Nucleocapsids are rod-shaped, with obvious regular surface structure. The size of the particle ranges from 92 to 160 nm long; usually straight (and fragile) with a clear modal length.

3.3.1.1 Molecular Structure

Total genome length is 9,400–10,800–13,500 nt. The genome is divided among more than one type of particle, i.e., 2 different types (or more containing either RNA-1 in the long particles, or RNA-2 in the shorter particles, or deletion mutants in the shortest particles).

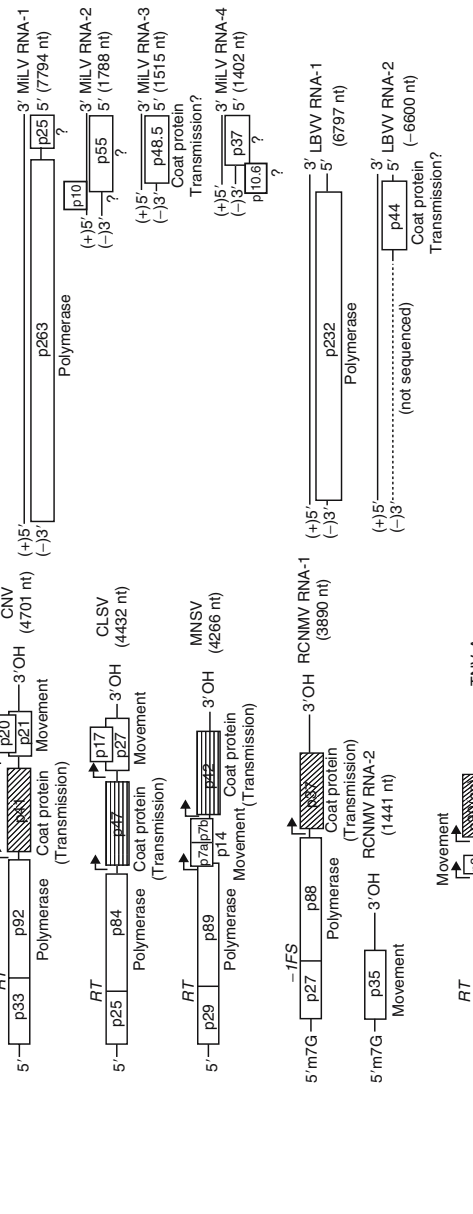

Fig. 2 Genome structures of viruses transmitted by olpidium. *Hatched boxes* highlight the coat protein ORF believed responsible for transmission specificity. Main functions of proteins are shown as well as expression strategies

3.3.2 Soilborne Wheat Mosaic Virus (SBWMV)

First described in 1919, SBWMV can cause severe stunting and mosaic in susceptible wheat, barley and rye cultivars. Currently, major gene resistance is incorporated into the most popular cultivars grown where SBWMV already occurs. This has resulted in a resurgence of research interest in the virus and control of the disease. *Soilborne mosaic* and *spindle streak mosaic* are both carried by a fungal vector called *Polymyxa graminis*. The virus particles are carried on or in the fungal zoospores (swimming spores).The fungus invades root hairs of the young wheat in the fall during periods of high soil moisture.

3.3.2.1 Molecular Structure

Soilborne wheat mosaic virus (SBWMV) 19K protein is a cysteine-rich protein (CRP) and shares sequence homology with CRPs derived from furo-, hordei-, peclu- and tobraviruses. SBWMV is a bipartite RNA virus and is the type member for the genus *Furovirus* (Mayo 1999). RNA1 encodes the viral replicase and putative viral movement protein (MP). The viral replicase is encoded by a single large open reading frame (ORF) and is phylogenetically related to the *Tobacco mosaic virus* (TMV) replicase (Shirako and Wilson 1993) The 3′ proximal ORF of RNA1 encodes a 37K MP that shares sequence similarity with the dianthovirus MP (An et al. 2003; Melcher 2000). SBWMV RNA2 encodes four proteins. The 5′ proximal ORF of RNA2 encodes a 25K protein from a nonAUG start codon and its role in virus infection is unknown. The coat protein (CP) ORF has an opal translational termination codon and readthrough of this codon produces a large 84K protein (Shirako 1998). The CP readthrough domain (RT) is required for plasmodiophorid transmission of the virus (Tamada et al. 1993) The 3′ proximal ORF of RNA2 encodes a 19K CRP. The present study shows that the SBWMV 19K CRP, when expressed from the PVX genome, functions as a pathogenesis factor and a suppressor of RNA silencing. The SBWMV 19K CRP, when it was expressed from the PVX genome, induced systemic necrosis on *Nicotiana benthamiana, N. clevelandii, C. quinoa*, and *C. amaranticolor*.

3.3.3 Bymovirus

Virions are not enveloped and are of more than one type of particle, and different in size and range. The nucleocapsids are filamentous, and usually flexuous with clear modal length. Virions contain two segments of linear positive-sense ssRNA. Total genome length is 8,500–10,000 nt. The 5′ end of the genome has a genome-linked protein (VPg) and the 3′ end has a poly (A) tract. Nongenomic nucleic acid is not found in the virions. The genome is divided among more than one type of particle, i.e., 2 different types. The RNAs of BaYMV have been sequenced (Kashiwazaki et al. 1990) and shown

to encode a genome organization similar to that on single component potyviruses but divided between the two segments.

Wheat spindle streak mosaic virus (WSSMV), which induces stunting and chlorosis is also temperature sensitive. A good diagnostic feature to distinguish between these two viruses is that WSSMV induces chlorotic streaks that are elongated and spindle-shaped and often have a dark green island in their center.

3.3.4 Benyvirus

Beet necrotic yellow vein virus (BNYVV), which is transmitted by *P. betae*, is the causal agent for rhizomania, a devastating disease of sugar beet found worldwide and in virtually every major sugar beet–growing areas in the United States. These viruses are positive strand RNA viruses belonging to nine genera. Plant viruses belonging to the genera *Bymo-*, *Beny-*, *Furo-*, *Peclu-*, and *Pomovirus* are vectored by plasmodiophorids.

3.3.4.1 Molecular structure

BNYVV is a positive strand RNA virus with four genome segments (Fig. 3). The presence of viral replicase inside *P betae* resting spores and zoospores may be evidence that BNYVV replicates inside its vector. According to this model, accumulation of viral replicase, coat protein, RTD, P25, and P31 proteins, which are expressed from the genomic RNAs, suggests that viral RNAs may be translated as *P. betae* progresses through its life cycle. The P42, P13, P15, and P14 proteins are produced from subgenomic RNAs derived from BNYVV RNA2. The subcellular accumulation patterns for BNYVV P25 and P31 were intriguing because these proteins are suggested to play significant roles in virus transmission and accumula-

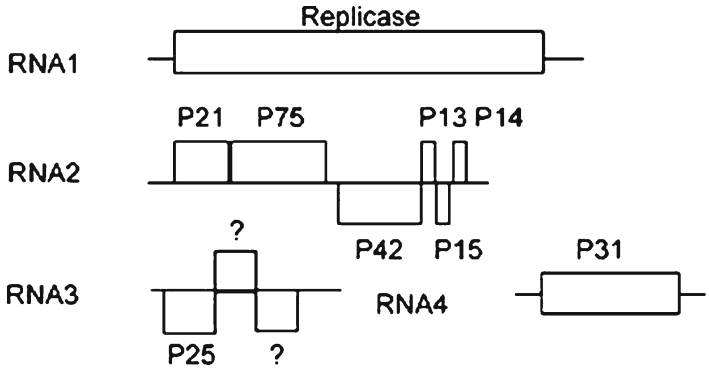

Fig. 3 Diagrammatic representation of the BNYVV genomes. *Lines* represent four genomic segments. *Boxes* represent coding regions. RNA2 is multicistronic. The 3′ ORFs (P42, P13, P15, P14) are expressed from subgenomic RNAs. The *names* for each coding sequence are provided above the *boxes*

tion in roots. P25 and P31 are the only proteins that localize to the zoospore nucleus P25 may play a role in symptom expression in plants.

3.3.5 Pomovirus

PMTV is soil-borne and has tubular rod-shaped particles. It is found in potato growing areas in Europe, Canada, South America and Asia that have a cool wet climate. It was recently identified in the USA (2002) and an extensive survey revealed that it was widespread in potato producing states. Typical tuber symptoms of brown lines, arcs or rings (spraing) in tuber flesh and raised external lines appear in the year of infection when the virus is transmitted to potato by the soil-borne 'fungus' *S. subterranea* f. sp. *subterranea*, the causal agent of powdery scab disease.

Virus-carrying *Spongospora* resting spores can remain viable in soil for many years and potatoes were infected with PMTV when planted in a field 18 years after potatoes were last grown. Particles of isolate T of potato mop-top furovirus (PMTV) contain three RNA species (6.5, 3.0 and 2.5 kb). RNA 2 (2,962 nt), which was sequenced, has noncoding regions of 368 nt and 285 nt at the 5′ end and 3′ end, respectively. From the 5′ end, the four open reading frames encode proteins of 51K, 13K, 21K and 8K. The 51K protein contains a putative NTP-binding motif and the 13K and 21K proteins each contain two hydrophobic regions separated by a hydrophilic region. The 8K protein is rich in cysteine. PMTV differs from other furoviruses in having a tripartite genome (Karen et al. 1994).

3.3.6 Watercress viruses

Watercress chlorotic leaf spot agent (WCLA), which has not been characterized (Tomlinson 1958), and *Watercress yellow spot virus* (WYSV), which has a polyhedral virion 37 nm in diameter (Walsh et al. 1989), are considered together because they have the same host, similar symptoms pattern, and perhaps the same vector. WCLA was transmitted when WCLA-*S. Subterranean nasturtii*-infected plants were placed in a container of water with healthy plants, but not in the presence of zinc, which would kill zoospores. In contrast, WYSV was transmitted from plants infected by *S. subterranea nasturtii* and WYSV in the presence of zinc in a similar trial. If *S. subterranea nasturtii* is the vector, it is the first plasmodiophorid to transmit a polyhedral virion.

4 Mycorrhiza and Viruses

Several studies have indicated that a host plant previously inoculated with a VA Mychorrhizal fungal symbiont exhibits increased resistant to different diseases. The effects of mycorrhizal infection on the production of three viruses in three

hosts were examined (Daft and Okusanya 1973). In tomato, the multiplication of *Tomato aucuba mosaic virus* was increased, while the amount of *Potato virus X* produced depended on the stage of the mycorrhizal development. The amount of extractable *Arabis mosaic virus* from petunia and strawberry plants was greater from mycorrhizal than from non-mycorrhizal plants. In electron microscopic studies, *Tobacco mosaic virus* was found to be restricted to plant cell cytoplasm; however it was more numerous in the cells that contain arbuscules (Arora et al. 1991). It was also found that *Piriformospora indica*, a root endophytic fungus induces resistance against *Pepino mosaic virus* (PepMV) in tomato (Fakhro et al. 2007).

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References

Adams MJ, Swaby AG, Jones P (1988) Confirmation of the transmission of *Barley yellow mosaic virus*. Ann Appl Virol 112:1331–41

Adams MJ, Antoniw JF, Mullins JGL (2001) Plant virus transmission by plasmodiophorid fungi is associated with distinctive transmembrane regions of virus encoded proteins. Arch Virol 146:1139–1153

An H, Melcher U, Doss P, Payton M, Guenzi AC, Verchot-Lubicz J (2003) Evidence that the 37 kDa protein of soil-borne wheat mosaic virus is a virus movement protein. J Gen Virol 84:3153–3163

Arora KD, Ajello L, Rai B, Elmer HM, Mukerji KG, Richard PE, Eivind BL, Bhatnagar D (1991) Handbook of Applied Mycology. Volume 1, 131–154

Barr DJS (1979) Morphology and host range of *Polymyxa graminis, Polymyxabetae*, and *Ligniera pilorum* from Ontario and some other areas. Can J Plant Pathol 1:85–94

Brown DJF, Robertson WM and. Trudgill D (1995) Transmission of viruses by plant nematodes. Annu Rev Phytopathol 33:223–249

Campbell RN (1962) Relationship between the lettuce big-vein virus and its vector, *Olpidium brassicae*. Nature 195:675–677

Campbell RN (1996) Fungal transmission of plant viruses. Annu Rev Phytopathol 34:87–108

Campbell RN Sim ST (1994) Host specificity and nomenclature of *Olpidium bornovanus* (= ←*Olpidium radicale*) and comparisons to *Olpidium brassicae*. Can J Bot 72:1136–1143

Campbell RN, Fry PR (1966) The nature of the associations between *Olpidium brassicae* and lettuce big vein and tobacco necrosis viruses. Virology 29:222–233

Campbell RN, Grogan RG (1963) Bigvein virus of lettuce and its transmission by *Olpidium brassicae*. Phytopathology 53:252–259

Campbell RN, Grogan RG (1964) Acquisition and transmission of lettuce bigvein virus by *Olpidium brassicae*. Phytopathology 54:681–690

Campbell RN, Sim ST, Lecoq H (1995) Virus transmission by host-specific strains of *Olpidium bornovanus* and *Olpidium brassicae*. Eur J Plant Pathol 101:273–282

Carrrington JC and Morris TJ (1984) Complementary DNA cloning and analysis of carnation mottle virus RNA. Virology 139:22–31

Chen JP, Swaby AG, Adams MJ, Yili R (1991) *Barley mild mosaic virus* inside its fungal vector, *Polymyxa graminis*. Ann Appl Biol 118:615–621

Clay CM, Walsh JA (1996) Watercress yellowspot virus (WYSV): cytopathic alterations suggest it is a member of the *Tombusviridae*. Proc 3rd Symp Int Work Group Plant Viruses Fungal Vectors, Dundee, Scotland

Coutts RHA, Rigden JE, Slabas AR, Lomonossoff GP, Wise PJ (1991) The complete nucleotide sequence of tobacco necrosis virus strain D. J. Gen Virol 72:1521–1529

Daft MJ, Okusanya BO (1973) Effect of endogone mycorrhiza on plant growth. V. Influence of infection on the multiplication of viruses in Tomato, Petunia and Strawberry. New Phytologist 72(5):975–983

Diao A, Chen J, Gitton F, Antoniw JF, Mullins J (1999) Sequences of European wheat mosaic 970. The relationship between *Cucumber necrosis virus* and its vector, *Olpidium cucurbitacearum*. Virology 42:204–211

Dick MW (2001) Straminopilous fungi: systematics of the peronsoporomycetes including accounts of the marine of novel filamentous morphology. J Gen Virol 75:3585–3590

Driskel B, Verchot-Lubicz J (2003) Interactions between *Soil-borne wheat mosaic virus* and its vector *Polymyxa graminis* may involve the viral movement protein. Am Soc Virol 22nd Annu Meet, University of California, Davis (Abstract)

Dubois F, Sangwan RS, Sangwan Norreel BS (1994) Immunogold labelling and electron microsopic screening of *Beet necrotic yellow vein virus* in the fungus *Polymyxa betae* infecting *Beta vulgaris* root cortical parenchyma cell. Int J Plant Sci 155:545–552

Fakhro A, Schwarz D, Franken P, Bargen SV, Bandte M, Büttner C (2007) Application of the endophyte *Piriformospora indica* in hydroponic cultures. *Tropentag* 2007 International Research on Food Security, Natural Resource Management and Rural Development Cuvillier Verlag Göttingen

Fry PR (1958) The relationship of *Olpidiumbrassicae* (Wor.) Dang. to the bigveindisease of lettuce. NZ J Agric Res 1:301–440

Gerhardson B, Insunza V (1979) Soil transmission of red clover necrotic mosaic virus. Phytopathol Z 94:67–71

Gray SM (1996) Plant virus protein involved in natural vector transmission Trends Microbiol 4:259–264.

Gray SM, Rochon DM (1999) Vectors of plant viruses. In: Granoff A, Webster R (eds) Encyclopedia of virology, vol 1. Academic, London, pp 1899–1910

Grogan RG, Zink FW, Hewitt WB, Kimble KA (1958). The association of *Olpidium* with the bigvein disease of lettuce. Phytopathology 48:292–296

Guilley H, Carrington, JC, Balazas E, Jonard G, Richards K and MorrisTJ (1985) Nucleotide sequence and genome organization of carnation mottle virus RNA. Nucl Acids Res 13:6663–6677

Harrison SC, Olson AJ, Schutt CE, Winkler FK, Brigogne G (1978) *Tomato bushy stunt virus* at 2.9 °A resolution. Nature 276:368–373

Hewitt WB, Raski DJ, Goheen AC (1958) Nematode vector of soil-borne fan leaf virus of grapevines. Phytopathology 48:586–595

Hogle JM, Maeda A, Harrison SC (1986) Structure and assembly of *Turnip crinkle virus*. I. X-ray crystallographic structure analysis at 3.2 °A resolution. J Mol Biol 191:625–638

Iwaki M, Hanada K, Ramirez A. Maria E, Onogi S (1987) Lisianthus necrosis virus, a new necrovirus from *Eustoma russellianum*. Phytopathology 77:867–870

Kakkani K, Sgro JY, Rochon D (2001). Identification of specific Cucumber necrosis Virus coat protein amino acids affecting fungus transmission and zoospore attachment. Virology 75:5576–5583

Kanyuka K, Ward E, Adams MJ (2003) *Polymyxa graminis* and the cereal viruses it transmits: a research challenge. Mol Plant Pathol 4:393–406

Karen P. Scott Satoshi K, Brian R and Bryan D. Harrison (1994) The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. J Gen Virol 75:3561–3568

Kashiwazaki S, Minobe Y, Omura T and Hibino H (1990) Nucleotide sequence of barley yellow mosaic virus RNA1: a close evolutionary relationship with potyvirus. J Gen Virol 71:2781–2790

Kashiwazaki S, Scott KP, Reavy B, Harrison BD (1995) Sequence analysis and gene content of potato mop-top virus RNA-3: further evidence of heterogeneity in the genome organization of furoviruses. Virology 206:701–706

Keskin B (1964) *Polymyxa betae* n. sp., ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrube. Arch Microbiol 49:348–374

Kruse M, Koenig R, Hoffmann A, Kaufmann A, Commandeur U (1994) Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. J Gen Virol 75:1835–1842

Kuwata S, Kubo S, Yamashita S, Doi Y (1983) Rod-shaped particles, a probable entity of *Lettuce big vein virus*. Ann. Phytopathol. Soc. Jpn. 49:246–251

Lange L, Insunza V (1977) Root inhabiting *Olpidium* species: the *O. Radicale* complex. Trans Br Mycol Soc 69:377–384

Lommel SA, Martelli GP, Russo M (2000) Family *Tombusviridae*. In: Van Regenmortel MHV, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch. DJ, Pringle CR, Wickner RB (eds) Virus taxonomy. Academic, San Diego, pp 791–825

Lot H, Campbell RN, Souche S, Milne RG, Roggero P (2002) Transmission by *Olpidium brassicae* of *Mirafiori lettuce virus* and *Lettuce big-vein virus*, and their roles in Lettuce Big-Vein etiology. Phytopathology 92:288–293

Macfarlane I (1982) Red clover necrotic mosaic virus (RCNMV). Rothamsted Exp Stn Rep 1981, p 190

Mayo MA (1999) Developments in plant virus taxonomy since the publication of the 6th ICTV report. Arch Virol 144:1659–1666

Mayo MA (2000) Genus *Varicosavirus*. In: Van Regenmortel MHV, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch. DJ, Pringle CR, Wickner RB (eds) Virus taxonomy. Academic, San Diego, pp 521–523

McLean MA, Campbell RN, Hamilton R I. and Rochon DM (1994) Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by *Olpidium bornovanus*. Virology 204:840–842

Melcher U (2000) The '30K' superfamily of viral movement proteins. J Gen Virol. 81(Pt 1):257–266.

Meulewaeter F, Seurinck J, van Emmelo J (1990) Genome structure of tobacco necrosis virus strain A. Virology 177:699–709

Miller JS, Damude H, Robbins MA, Reade RD, Rochon DM (1997) Genome structure of *Cucumber leaf spot virus*, sequence analysis suggests it belongs to a distinct species within the *Tombusviridae*. Virus Res 52:51–60

Milne RG. (2002) Ophioviruses: hiding in the background, emerging from the underground. In: CM Rush, U Merz (eds) Proc 5th Symp Int Work Group Plant Viruses Fungal Vectors. Inst Plant Sci, Swiss Fed Inst Technol, Zurich, pp 68–71

Morikawa T, Nomura Y, Yamamato T, Natsuaki T (1995) Partial characterization of virus-like particles associated with Tulip mild mottle mosaic. Ann Phytopathol Soc Jpn. 61:578–581

Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, et al. (1995) Virus taxonomy classification and nomenclature of viruses. Arch Virol Suppl 10

Mutasa ES,Ward E, Adams MJ, Collier CR, Chwarszczynska DM, Asher MJC (1993) A sensitive DNA probe for the detection of *Polymyxa betae* in sugar beet roots. Phys Mol Plant Pathol 43:379–390

Naum-Ongania G, Gago-Zachert S, Pena E, Grau O, Garcia ML (2003) *Citrus psorosis virus* RNA 1 is of negative polarity and potentially encodes in its complementary strand a 24K protein of unknown function and 280K putative RNA dependent RNA polymerase. Virus Res 96:49–61

Oda Y, Saeki K, Takahashi Y, Maeda T, Naitow H (2000) Crystal structure of *Tobacco necrosis virus* at 2.25 resolution. J Mol Biol 300:153–169

Pirone TP, Blanc S (1996) Helper dependent vector transmission of plant viruses. Annu Rev Phytopathology 34:227–247

Rao AS, Brakke MK (1969) Relation of soil-borne wheat mosaic virus and its fungal vector, *Polymyxa graminis*. Phytopathology 59:581–587

Reade R, Delroux K, Macdonald K, Sit TL, Lommel SA, Rochon D (2001) Spontaneous deletion enhances movement of a *Cucumber necrosis virus* based chimera expressing the red clover necrotic mosaic virus movement protein gene. Mol Plant Pathol 2:13–21

Reade R, Miller J, Robbins M, Xiang Y, Rochon D (2003) Molecular analysis of the *Cucumber leaf spot virus* genome. Virus Res 91:171–179

Reade R, Wu Z, Rochon D (1999) Both RNA rearrangement and point mutation contribute to repair of defective chimeric viral genomes to form functional hybrid viruses in plants. Virology 258:217–231

Rochon DM, Tremaine JH (1989) complete nucleotide sequence of the cucumber necrosis virus genome. Virology 71:251–259

Rochon D, Kakani K, Robbins M, Reader R (2004) Molecular aspects of plant virus transmission by olpidium and plasmodiophoroid vectors. Annu Rev Phytopathol 42:211–241

Roggero P, Ciuffo M, Vaira AM, Accotto GP, Masenga V, Milne RG (2000) An *Ophiovirus* isolated from lettuce with bigvein symptoms. Arch Virol 145:2629–2642

Rysanek P, Stocky G, Haeberle AM, Putz C (1992) Immunogold labelling of *Beetnecrotic yellow vein virus* particles inside its fungal vector, *Polymyxa betae*. Agronomy 12:651–659

Sasaya T, Kishikawa K, Koganezawa H (2001) Nucleotide sequence of the coat protein gene of *Lettuce big-vein virus*. J Gen Virol. 82:1509–1515

Shirako Y (1998) Non-AUG translation initiation in a plant RNA virus: a forty-amino-acid extension is added to the N terminus of the soil-borne wheat mosaic virus capsid protein. J Virol 72:1677–1682

Shirako Y, Wilson TM (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. Virology 195:16–32

Stobbs LW, Cross GW, Manocha MS (1982) Specificity and methods of transmission of cucumber necrosis virus by *Olpidium radicale* zoospores. Can J Plant Pathol 4:134–142

Tamada T, Schmitt C, Saito M, Guilley H, Richards K, Jonard G (1996) High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae*. J Gen Virol 77:1359–1367

Temmink JHM, Campbell RN, Smith PR. (1970) Specificity and site of in vitro acquisition of tobacco necrosis virus by zoospores of *Olpidium brassicae*. J Gen Virol 9:201–213

Thouvenel JC, Fauquet C (1981) Further properties of peanut clump virus and studies on its natural transmission. Ann Appl Biol 97:99–107

Tomlinson JA (1958) Crook root of watercressIII. The causal organism *Spongospora subterranea* (Wallr.) Lagerh. f.sp. *nasturtii* f. sp. nov. Trans Br Mycol Soc 41:491–498

Tomlinson JA, Garrett RG (1964) Studies on the lettuce big-vein virus and its vector *Olpidium brassicae* (Wor.) Dang. Ann Appl Biol 54:45–61

Tomlinson JA, Faithful EM, Webb MJW, Fraser RSS, Seeley ND (1983) Chenopodium necrosis: a distinctive strain of tobacco necrosis virus isolated from river water. Ann Appl Biol 102:135–147

Torok VA, Vetten HJ (2002) Characterization of an ophiovirus associated with *Lettuce ring necrosis*. In: Lesemann DE, Vetten HJ (eds) Joint Conf Int Working Groups on Legume and Vegetable Viruses, Bonn 4–9 August 2002, Abstract p 4

Vaira AM, Accotto GP, Constantini A, Milne RG (2003) The partial sequence of RNA1 of the ophiovirus *Ranunculus white mottle virus* indicates its relationship to rhaboviruses and provides candidate primers for an ophiovirus-specific RT-PCR test. Arch Virol 148:1037–1050

Van der Wilk F, Dullemans AM, Verbeek M, van den Heuvel JFJM (2002) Nucleotide Sequence and genomic organization of an ophiovirus associated with lettuce big-vein disease. J Gen Virol 83:2869–2877

Verchot-Lubicz JV, Rush CM, Payton M, Colberg T (2000) Beet necrotic yellow vein virus accumulates inside resting spores and zoosporangia of its vector *Polymyxa betae BNYVV* infects *P. betae*. J Virol 4:37

Vetten HJ, Lesemann DE, Dalchow J (1987) Electron microscopical and serological detection of virus-like particles associated with Lettuce big-vein disease. J Phytopathol 120:53–59

Walsh JA, Clay CM, Miller A (1989) A new virus disease of watercress in England. EPPO Bull. 19:463–70

Ward E, Adams MJ, Mutasa ES, Collier CR, Asher MJC (1994) Characterization of Polymyxa species by restriction analysis of PCR-amplified ribosomal DNA. Plant Pathol 43:872–877

Intertwined Existence: The Life of Plant Symbiotic Fungi in Agricultural Soils

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1 Introduction

Arbuscular mycorrhizal (AM) fungi are root endophytes that have evolved with land plants for over 400 million years (Pirozinsky and Dalpé 1992). Through time, fungal endophytes and plants have become interdependent. Plants rely largely on fungi for soil nutrient uptake and some fungi even became obligate biotrophs unable to exist without a living host plant.

Plants and fungi have coevolved within relatively stable ecosystems, in very successful symbiosis at times, as illustrated by the widespread occurrence of AM mycorrhizae. Cropping systems are typically highly disturbed systems in which cropping practices often harm symbiotic fungi or their host plants. We know that the full potential of the AM symbiosis is rarely realized in cultivated fields, leading to inefficiencies in the function of these ecosystems. Other fungal endophytes of crop roots were overlooked and their role in plant fitness remains largely obscure, although they may be important in ecosystem function. Disruption in the proper function of plant symbioses may lead to inefficiencies in the function of cultivated soils. With the impoverishment of cultivated soils in poor countries and environmental quality degradation due to the loss of residual fertilizer to the environment in economically favored countries, it becomes urgent to improve the nutrient use efficiency of crop production. Improving the effectiveness of the AM symbiosis in cultivated fields would certainly be an important step toward this goal. Improving plant symbiosis effectiveness requires a good understanding of the conditions specific to cultivated soils that are influencing beneficial plant symbiosis. Therefore, the goal of this chapter is to consider the factors influencing the symbioses formed between plants roots and soil fungi, most importantly AM fungi.

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2 Fertilizer Impact on Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal fungi enhance plant growth and uptake of nutrients, particularly those with reduced mobility in soil, such as phosphorus (P) (Al-Karaki and Clark 1999; Jeffries et al. 2003; Kahiluoto et al. 2001). The benefits of AM associations are generally high on soils with low P content and are easily demonstrated in controlled pot experiments. Although AM fungi are undoubtedly an important component of soils quality, their contribution to agricultural production in field soils has been technically difficult to evaluate (Fitter 1985; Jakobsen 1994). Arbuscular mycorrhizal fungi are soil dwellers and obligate biotrophs, hence they are difficult to study. As a result, only very limited recommendations can be made to farmers at this time to help them manage these fungi in agricultural soils, and cropping practices are almost always developed without consideration for AM fungi.

Mineral fertilizers are used in agricultural production systems to optimize crop nutrition and productivity; their effects on the soil biological quality are generally overlooked. Fertilization of field soil may have important negative effects on AM fungi. The most important of these effects is related to residual P build-up in soils. Application of P fertilizer increases P availability and reduces crop plants' dependence on AM associations. High soil P fertility leading to high P concentration in plant tissues generally decreases the level of root colonization by AM fungi in various plant species (Koide and Li 1990; Ryan et al. 2000; Baath and Spokes 1989; Covacevich et al. 2007). Phosphorus is very reactive in soil and much of the P fertilizer that is applied to soils become bound on the soil solids before being used by crop plants. Repeated applications of P fertilizer to agricultural soils result in the buildup of residual P along with soil P fertility, which represses AM colonization of plants (Jokela 1992; Thomson et al. 1992). With reduced AM development, plants dependence on P fertilizer increases. In some intensively managed agricultural fields, soil P level has increased to the point of being threatening to the environment. Phosphorus movement to water bodies through wind or water erosion lead to eutrophication.

The level of mycorrhizal dependency of a host plant varies with P availability. Generally, root colonization is reduced in plants with high P status, whereas low P levels enhance root colonization. Higher root colonization in plants with relatively low tissue P concentration conditions has been explained by more abundant release of carbon-rich root exudates in these plants, making them better hosts than well fed plants (Nagahashi and Douds Jr 2000; Nagahashi et al. 1996; Sylvia and Neal 1990). The biological activity of root exudates from P-rich and P-deficient plants was shown to differ. While the root exudates of P-deficient plants stimulate hyphal branching, hyphal growth (Nagahashi et al. 1996; Tawaraya et al. 1995) and root colonization (Pinior et al. 1999; Tawaraya et al. 1998), root exudates of P-rich plants have only weak stimulation activity on hyphal growth (Tawaraya et al. 1995) and do not stimulate root colonization (Tawaraya et al. 1998). Phosphorus fertilization decreases the development of external hyphae (Bruce et al. 1994), spore abundance

(Martensson and Carlgren 1994; Miranda and Harris 1994), arbuscules number and size (Mosse 1973) and changes the species composition of AM fungal populations (Johnson 1993).

Plant physiology largely controls the development of the AM symbiosis. Arbuscular mycorrhizae are more developed when they are required for P uptake, under low soil P fertility, but plants invest less carbon and energy in their development and maintenance under conditions of nutrient abundance where AM fungi bring them little immediate benefits. We know that the decrease in AM colonization at high P is mediated through reduced carbon allocation by host to roots and AM fungi (Lu et al. 1994; Olsson et al. 1997; Valentine et al. 2001), but the exact mechanisms involved remain obscured. Phospholipids make up most of cell membranes and a decrease in cell membrane permeability in plants with high P concentrations was proposed to explain lower root colonization in these plants (Smith and Read 1997). Different AM fungal species may respond differently to increase in P availability or decrease in carbon allocation resulting in shifts in AM fungal community composition with changes in soil fertility. Some AM fungal species such as *Scutellospora calospora* are sensitive to low carbon allocation to roots and these species may be eliminated first by P fertilization (Thomson et al. 1986).

Excessive soil P fertility is deleterious to AM associations and AM fungi, which depend on host plants for their carbon nutrition. But at the other end of the soil fertility spectrum, under conditions of extreme soil infertility, the AM symbiosis does not develop well. The application of moderate amounts of P fertilizers to very deficient soils increases the densities of AM fungal spores and hyphae in the soil (Thomson et al. 1992), and the level of host plant root colonization (Bolan et al. 1984; Xavier and Germida 1997). A minimum amount of P must be present in soil. The AM symbiosis, by contrast to N_2-fixing symbioses, does not import P in the system, but rather, improves plants' ability to extract soil P. AM fungi have nutrient requirements of their own that must also be fulfilled for them to grow (Balser et al. 2005).

The typical soil P fertility response curve of AM development is convex, with low levels of development in conditions of insufficient and excessive P fertility on both sides of a sufficiency range corresponding to effective symbiosis development. The soil P availability threshold beyond which AM development is reduced varies with crops and crop genotypes. Good crop yields and good AM development are not mutually exclusive, and these two desirable outcomes may occur concurrently in the sufficiency range of the P fertility response curve. For example, highest relative wheat shoot dry matter yield was obtained at 15.5 mg P kg^{-1} at which average AM colonization was 39% (Covacevich et al. 2007). Arbuscular mycorrhizal root colonization in wheat decreased with an increase in soil Bray-P level up to 27 mg kg^{-1}, a level beyond which AM root colonization remained constant at 10%. Thus, in order to obtain the maximum benefit from mycorrhizal association, fertilizer application may be planned in a way that does not exceed the soil P threshold values for crops. It is important here to remember that the benefits derived from the AM symbiosis are not only nutritional but also include the maintenance of soil biological and structural quality.

Arbuscular mycorrhizal development in crops does not solely depend on the crop genotype and amount of P fertilizer applied. The source of P fertilization and the method of application may also influence AM development. In band applied P fertilizers, high P concentration restricts root colonization in roots present in the band, but the roots growing outside the band are much less affected. Thus, reduced colonization is predominantly localized in roots that actually contact applied P (Lu et al. 1994). Reduction in root colonization can be substantially limited by banding fertilizer. Lower root colonization in furrow applied P as compared to side band placement observed in corn roots (Bittman et al. 2006) was ascribed to more contact of roots in the seed furrow than in the side band. Furthermore, localized application of P is more effectively used by the crop as a smaller proportion of the P applied will react with soil P fixation sites. Lower rates of P fertilizer are required with localized application, and lesser amounts of residual P will be left after band application than broadcast of P fertilizers.

Soluble forms of P fertilizers such as ammonium phosphate or triple superphosphate increase the concentrations of phosphate ions in the soil solution and plant tissue thereby decreasing AM development. Less soluble forms of P fertilizers, like compost or rock phosphate, are less inhibitory as available P is released slowly, and concentration in fertilized crop plants may not reach a level inhibitory to mycorrhizal association (Toro et al. 1997). In contrast to soluble fertilizers that create a peak of fertility after application, organic manures may enhance AM development and the number of AM fungi propagules in soil (Harinikumar and Bagyaraj 1989; Muthukumar and Udaiyan 2002). The role of organic amendments may be of particular importance in agroecosystems where these are used on a regular basis as in organic agriculture. Increased root colonization has been observed under organic as compared to conventional farming systems (Ryan et al. 1994; Sattelmacher et al. 1991). The nutrients in organic manures are released at a slower rate and over a longer period of time, explaining their relatively mild impact on AM fungal colonization of roots as compared to the high-grade mineral fertilizers commonly used in intensive agriculture (Joner 2000).

The influence of N fertilizers on mycorrhizal association is less consistent that that of P, perhaps because it has been less studied and many of the observations gathered were from experiments testing the effect of P levels on the AM symbiosis. Experimental evidence indicates that soil N enrichment may increase, decrease or have no effect on root colonization by AM fungi (Azcon et al. 1982; Hayman 1982; Hepper 1983; Johnson et al. 1984). Like P, small and moderate amounts of N may increase root colonization (Eom et al. 2001) while higher rates of N applications decrease root colonization (Egerton-Warburton and Allen 2000). Liu et al. (2000) observed highest root colonization of corn at intermediate level of N application. Similarly, at a field site with high availability of soil P, plants from N-deficient plots were intensely colonized by AM fungi, whereas in plots with comparable P levels but higher soil and plant N concentrations, AM colonization rates were significantly lower (Blanke et al. 2005). It appears from such results that the ratio of available N and P in the soil, rather than the P level alone, is the important factor determining AM development (Johnson et al. 2003; Liu et al. 2000; Miller et al.

2002). A N-limited plant will have high P concentration at moderate to low P levels. As leakiness of the plasmalemma of root cortical cells is regulated by levels of cellular P, the carbon allocation to AM fungal growth and maintenance can be reduced in small N-limited but P-rich plants (Guttay 1983; Hepper 1983). Furthermore, AM fungi have nutrient requirements of their own. They need sufficient amounts of N for the synthesis of protein and chitin, the main constituents of their cell walls (Bethlenfalvay and Ames 1987). The nutritional requirements of different AM fungi vary; results show that applications of high rates of both N and P fertilizers for several years can favor communities of AM fungi that are less beneficial for plants than the communities from unfertilized soils (Corkidi et al. 2002; Johnson 1993).

The life of AM fungi and crop plants are intertwined. Fertilization of the crop does affect their AM fungal partners. While applications of moderate amounts of N and P fertilizers to soils of low fertility can be beneficial or noninhibitory for both the plants and the AM fungi, higher rates of mineral fertilizers generally depress AM fungi and even the plants when toxic levels are reached. Harm to the environment related to fertilizer use, such as eutrophication of water bodies, soil structural degradation and greenhouse gas production, occurs well before these later levels are reached. Thus, sustainable fertilization regimes are those permitting AM development and the contribution to crop nutrition.

3 Soil Tillage

Soil tillage is widely practiced in crop production as a mean for seed bed preparation, weed control, incorporation of crop residues and organic amendments, or to hasten soil drying and warming after snow melt. Soil disturbance due to tillage operations generally reduces AM colonization of roots (Lekberg and Koide 2005) and the abundance of AM hyphae in soil, which reduces P uptake by crops and, sometimes, crop yield (Kabir 2005; Miller 2000; Miller et al. 1995). Soil tillage disrupts AM hyphal networks, fragmenting AM mycelia. Arbuscular mycorrhizal fungi are biotrophs and their life depends on a physical connection to a plant root; in tilled soil, several mycelial fragments may never reconnect to a plant. The content of these hyphal pieces are, then, subtracted from soil AM networks and added to the pool of dead soil organic matter.

In addition to weakening AM fungal networks, soil tillage has a profound effect on the soil environment and other soil organisms, including those interacting with AM fungi. Tillage influence on soil quality is both direct through soil mixing and soil aggregate disruption, and indirect through a negative impact on AM fungi. Tillage reduces the proportion of fungi, most importantly AM fungi, to bacteria (Allison et al. 2005). AM fungi are an important factor of soil aggregate stabilization (Abiven et al. 2007; Six et al. 2004); the reduction of their proliferation compounds the negative effect of tillage on the physical disintegration of aggregates and mineralization of the organic matter cementing them. Tillage disrupts soil

aggregates exposing fresh organic matter to the activity of decomposers (Blanco-Canqui and Lal 2004; Manlay et al. 2007). This reduces soil organic matter level along with soil microbial biomass, which is usually correlated with soil organic matter content (Brady and Weil 2001), and has a negative influence on soil water relation and fertility properties that are also related to soil organic matter level (Loveland and Webb 2003). Borie et al. (2006) measured increased soil organic matter, nitrogen (N), sulphur (S), P and pH levels under no-till or reduced tillage. Albuquerque et al. (2005) observed a negative effect of tillage on soil aggregate stability, water storage capacity, soil organic carbon and P content.

Modification of the soil conditions by tillage influences the soil microbial community, which is interacting with AM fungi. Certain soil fungi influence AM fungi spore germination (McAllister et al. 1994), development and function (Martinez et al. 2004), and presymbiotic behavior (Fracchia et al. 1998). Several free-living (Babana and Antoun 2005; Babana and Antoun 2006; Fester et al. 1999; Gamalero et al. 2004; Khan 2005; Ratti et al. 2001; Srinath et al. 2003; Vivas et al. 2003) and symbiotic bacteria (Antunes et al. 2006b) influence root colonization by AM fungi. Thus, modification of the soil environment by tillage can influence AM fungi both directly and indirectly, via the biological interactions taking place in soil.

The end result of the combined effect of physical hyphae disruption, soil environment modification and change in soil microbial community structure is often a change in the taxonomic composition of AM fungi community. Although there are reports of *Glomus* species being favored by no-tillage management (Douds et al. 1995; Hamel et al. 1994), species of the Glomeraceae are generally seen as relatively more resilient to soil disturbance than species of the Gigasporaceae (Castillo et al. 2006; Jansa et al. 2002; Jansa et al. 2003; Lovera and Cuenca 2007; Schalamuk et al. 2006). The susceptibility of the Gigasporaceae has been explained by the slow growth of species in this family and to a limited ability to form anastomosis between different hyphae, unlike species of the Glomaceae (Antunes et al. 2006a). Anastomoses have been mainly observed in species of *Glomus* (Bianciotto et al. 2004), but were also seen in *Scutellospora reticulata* (de Souza and Declerck 2003), *Gigaspora margarita*, and *Gigaspora rosea* (de la Providencia et al. 2005). Tillage favored *Glomus mosseae, Glomus* sp 6., *Scutellospora pellucida* (Menéndez et al. 2001) and species of the *Glomus etunicatum* group (Douds et al. 1995), while absence of disturbance favored *Acaulospora denticulata, Entrophospora* spp., *Glomus* spp. 1–5, *Glomus aggregatum, Glomus microaggregatum, Glomus coremioides* (Menéndez et al. 2001) and species of the *Paraglomus occultum* group (Douds et al. 1995; Galvez et al. 2001). The particular sensitivity of *Acaulospora* morphotypes to soil disturbance was reported (Merryweather and Fitter 1998). It is unclear at this time if the ability to form anastomoses is an isolate-specific feature, or a universal phenomenon occurring to different extents in different isolates or under different conditions; but it is clear that some AM fungal isolates are somehow more susceptible to disturbance although they form anastomoses. Anastomosis formation perhaps helps AM fungal networks to reconnect after tillage-induced fragmentation.

Fungi of the Glomeraceae possess intraradical vesicles that remain viable in dead root fragments. These vesicles allow the fungi to spread more rapidly and to

better recover from a tillage operation than fungi of the Gigasporaceae, which possess non-infective extraradical auxiliary cells and no intraradical vesicles. Fungi of the Gigasporaceae may rely only on spore production for propagation (Biermann and Linderman 1983). Early sporulation could also be an important attribute of species adapted to tilled soils, as illustrated by *Glomus scintillans*, an early sporulator, that became dominant in plowed soils of Columbia (Sieverding 1991).

The life-strategy of AM fungal species largely determines their success in tilled soils. Species producing abundant infective propagules between tillage operations are more likely to contact and form mycorrhizal associations with crops emerging from tilled soils. Fast-growing species with infective extraradical hyphae will spread rapidly within a crop. Prolific and fast-growing AM fungi are seen as better adapted to tilled soils than slow growers. Such fungi may constitute a larger carbon drain than fungi with less exuberant habit, however; AM fungi in tilled soils are, perhaps, inferior mutualists (Johnson et al. 1992; Johnson and Pfleger 1992).

Tillage influences the AM community structure of soils, but may also influence AM fungal biodiversity. Biodiversity was sometimes increased by tillage (Castillo et al. 2006), sometimes not influenced (Jansa et al. 2002; Schalamuk et al. 2006), and sometimes decreased (Kabir 2005). This variable effect of tillage on AM fungal biodiversity may depend on the length of the period over which tillage is applied, depth of tillage, and depth of sampling. Oehl (2005) suggested that AM fungal inoculum located below the soil zone affected by agricultural practices serves as a precious source of biodiversity that buffers the effect of changes in practices on the AM fungal community of the upper soil layer.

4 Pest Control

The practice of monoculture cropping, or in some cases seeding a few different species together, has led to a decrease in biodiversity, or at least a decrease in "desirable" diversity within agricultural areas. This has led to increases in pesticide use to reduce weeds, plant diseases and insect pests in agricultural fields. These chemicals can have an effect on nontarget organisms, one group of which are arbuscular mycorrhizal fungi. It has been suggested that nontarget detrimental effects on AM (Trappe et al. 1984; Vyas and Vyas 2000) may diminish the benefits of pesticides for controlling crop pests. The effects can vary greatly and results found within the literature are at times contradictory (Menge et al. 1979; Trappe et al. 1984; Vyas and Vyas 2000).

Impacts of herbicides on arbuscular mycorrhizae have been shown in many studies. Physiological changes in the potential host plant due to herbicides can create the conditions for AM to thrive (Nasr 1993). One study that brings this clearly into focus is the mycorrhization of a "non-host" plant, *Chenopodium quinona* (Schwab et al. 1982). Simazine was applied in sublethal doses to the plant and this led to an increase in root exudation, which was felt to be responsible for formation of AM.

Other studies have found that herbicides have little impact on AM (Girvan et al. 2004). Preseeding field application of glyphosate was found to have no effect on AM colonization of soybean. However, in vitro high levels of the herbicide reduced germination and growth of spore germ tubes (Malty et al. 2006).

Some herbicides have been found to be detrimental to AM formation. In a pasture, an introduced species, *Bromus tectorum*, was found to significantly lower AM root colonization at higher rates of tebuthiuron application compared to low rates or the control (Allen and West 1993). The differences between herbicide rates were not found 2 months later, though AM spores were significantly less abundant in *B. tectorum* rhizosphere at the same time. Changjin and Bin (2004) found all six herbicides they tested decreased AM colonization, hyphal enzyme activities, hyphae in the soil and reduced the biomass of the host plant, maize.

The decrease in mycorrhization is not always due to direct impacts on the plant or the AM under study. In a trial with conventional production practices both sugar beet and maize had reduced AM after herbicide application (Baltruschat 1985). However, when the herbicides were tested on these crops in a greenhouse trial there was no reduction in AM. The reason proposed for the reduction of AM colonization under field conditions was due to elimination of weeds that may be hosts to AM, and the only inoculum for the crops were spores remaining in the soil. Weed control measures including herbicide treatments also affected mycorrhizal status and AM species composition in grapevine (Baumgartner et al. 2005) and cassava (Sieverding and Leihner 1984).

The AM connections between target weeds and crops have been demonstrated in some studies. The herbicide bentazon when applied to soybean and cocklebur was shown to reduce the AM-colonized root length of the cocklebur by 43%, but there was little effect on the soybean (Bethlenfalvay et al. 1996). As the susceptible cocklebur succumbed to the bentazon application, an AM-mediated flux of nutrients occurred from weed to crop (Bethlenfalvay et al. 1996). A similar response was found when chlorsulfuron was applied to soybean and a weed species (Mujica et al. 1998). Diclofop was found to inhibit AM root colonization in wheat, however, when grown with ryegrass (susceptible to diclofop) wheat growth and yield were enhanced (Rejon et al. 1997). This wheat growth increase was attributed to interplant AM associations with the wheat becoming a stronger sink for nutrients than the ryegrass. As found in other studies (Siquiera et al. 1991) and illustrated above, herbicide effects on AM are complex.

Fungicides are often looked upon as potentially having the largest impact on arbuscular mycorrhizae and possibly the most deleterious effects. One study shows that there is a variable response of arbuscular mycorrhizae to systemic fungicides (von Alten et al. 1993). They found that two fungicides increased AM root colonization of the host barley, two decreased the colonization and five had no effect. Similarly, Plenchette and Perrin (1992) found that systemic fungicides decreased or had no effect on mycorrhization of leek while non-systemic fungicides affected wheat. They also found that one fungicide stimulated mycorrhizal development. The literature is full of examples showing that effects are quite variable (Diedhiou et al. 2004; Jabaji-Hare and Kendrick 1987; Kjoller and Rosendahl 2000; Kling and

Jakobsen 1997; Salem et al. 2003; Schreiner and Bethlenfalvay 1997; Sukarno et al. 1998; von Alten et al. 1993) and in some cases contradictory (Trappe et al. 1984; Vyas and Vyas 2000).

In addition to fungicide type, the placement (in space or time) of fungicide can be very important and again contradictory in the effects on arbuscular mycorrhizae. Seed treatments have been found to be both detrimental and beneficial to AM colonization depending on fungicide type and plant cultivar (Jayaraman and Kumar 1995). When PCNB (pentachloronitrobenzene) was applied at the field rate in soil that grew cotton, there were initially reductions in AM root colonization, plant biomass and plant P content. However after 6 weeks, there were no treatment differences (Pattison et al. 1997). The fact that some fungicides do not have deleterious effects can be exploited to aid in the production of AM for commercial inoculants (Kumar and Bagyaraj 1999). Some fungicides are listed as compatible with inoculants (e.g., Myke®; Premier Tech Biotechnologies, Riviere-du-Loup, Quebec).

Insecticides as well as nematicides and arachnicides have also been studied for their effects on AM. Similar to both herbicides and fungicides, the results vary. *Cynara cardunculus* inoculated with *Glomus mosseae* and treated with one of two insecticides was found to have lower AM colonization. However, when inoculated with a local *Glomus* sp. strain and treated with pesticides there was no effect (Marin et al. 2002). The use of select insecticides has been found to have little effect on AM P uptake (Schweiger and Jakobsen 1998) or root colonization (Wan and Rahe, 1998). Chlorpyrifos was found to increase AM fungal diversity when applied to mixed grassland (Vandenkoornhuyse et al. 2003); this was attributed to a decrease in mesofaunal populations and hence a decrease in predation on AM.

5 The Selection of Rotation Crops

The selection of crops grown in rotation on land may have a large influence on the AM fungal population. The choice of crop sequences in rotations including host and nonhost plant species, are critical factors affecting the development, activity and diversity of AM fungi (Castillo et al. 2006; Douds Jr and Millner 1999; Oehl et al. 2005; Plenchette et al. 2005). Growing a nonmycorrhizal crop, such as canola and other crop plants of the Brassicaceae and Chenopodiaceae, can be more detrimental to a subsequent highly mycorrhizal-dependant crop than either tillage or P fertilization (Gavito and Miller 1998). Crops with low level of mycorrhizal dependency and development can also negatively impact subsequent AM crops (Gosling et al. 2006; Plenchette et al. 2005).

Plant AM colonization level and AM fungal spore density were reduced in soil growing field crops for some 30 years in southwest China (Lingfei et al. 2007). This study extends and confirms the finding by Helgason (1998) that AM fungal diversity is low in arable crops (Daniell et al. 2001). The impact of crop production can be compounded by the effect of monoculture, and further decrease the abundance of AM fungal spores (Rao et al. 1995) and shift the AM fungal species composition of

the community in favor of less mutualistic species (Douds Jr and Millner 1999; Johnson et al. 1992).

Hijri et al. (2006), by contrast, reported that low-input agriculture involving crop rotation may provide better conditions for healthy expression of the AM symbiosis by preserving AM fungal diversity, and preventing the selection for the AM fungal taxa that are inferior mutualists, but tolerate high nutrient levels. The use of legumes, which were found to enhance soil AM fungal communities and mycorrhizal potential, has been suggested to restore AM fungal biodiversity (Duponnois et al. 2001). The use of winter wheat cover crops was proposed to increase soil mycorrhizal potential and the growth and yield of maize, the following season (Boswell et al. 1998). Alternatively, the careful management of some existing mycorrhizal weed populations, such as dandelion, could be profitable both financially and ecologically (Kabir and Koide 2000).

The establishment of a barley crop on an old meadow in Canada was proposed as the most likely factor explaining the change in AM fungal species frequency in soil (Hamel et al. 1994). A change in plant host can cause some AM fungal species to decline or cause others to increase in abundance. The quality of soil microbial community should be considered in the assessment of the effect of agriculture on biodiversity, as intensive agriculture may be operating at minimum levels of diversity for at least one key functional group: the AM fungi (Helgason et al. 1998).

6 Spatial and Seasonal Variations Influencing Symbiotic Fungi in Pastures

Hay and pasture are important crop productions in many agricultural systems. In the grasslands, symbioses with dark septate endophytic (DSE) and AM fungi are common. The use of molecular techniques (Jumpponen and Johnson 2005; Vandenkoornhuyse et al. 2002) and culture based methods (Wilberforce et al. 2003) has provided an overview of the large genetic diversity of these fungi in grass roots. AM fungi can colonize the roots and provide different benefits to the majority of cultivated plant species. In grasses of many ecosystems, DSE are as natural as AM fungi (Table 1). Recent reviews have presented the potential role of DSE (Mandyam and Jumpponen 2005) and their structural differences with AM fungi (Brundrett 2004; Jumpponen 2001). Overall, little is known about how these fungi interact or their consequences on plant fitness.

To understand the ecological value of grass–DSE–AM fungi associations, consideration must be given to variations in time and space in the structure and function of these multipartite associations. Their intertwined nature and success can be better understood by considering the development of "adaptive transient symbioses". In environments with marked seasonality, plant roots can take advantage of root endophytes biodiversity through the development of adaptive transient symbioses that improve their capacity to use soil resources, which fluctuate seasonally.

Table 1 Reports on the simultaneous colonization of grass roots by DSE and AM fungi in different environments

Plant species	Environment	Reference
Cynodon dactylon (L.) Pers	Kunming, southwest China	(LingFei et al. 2005)
Cyperus rotundus L		
Digitaria cruciata (Nees ex Steud.) Camus		
Digitaria ischaemum (Schreb.) Schreb. ex Muhl		
Paspalum distichum L		
Plantago asiatica L		
Poa annua L		
Taraxacum mongolicum Hand-Mazz		
Trifolium repens L		
Agrostis stolonifera L	Wetlands calcareous fens	(Weishampel and Bedford 2006)
Bromus ciliatus L		
Calamagrostis canadensis (Michx.) Beauv		
Glyceria striata (Lam.) A.S. Hitchc		
Poa laxa Haenke.	Central and northern calcareous Alps, Austria	(Haselwandter and Read 1980)
Perotis indica (L.) Kuntze.	Western ghats, southern India.	(Muthukumar et al. 2006)
Cyperus conglomerates Linn	Cholistan desert, Pakistan	(Chaudhry et al. 2006)
C. rotundus Linn		
Cymbopogon jwarancusa (Jones Schult.		
Cenchrus ciliaris Linn		
Ochthochloa compressa (Forsskal.) Hilu.		
Saccharum bengalense Retz.		
Sporobolus iocladus (Nees. Ex Trin) Nees		
Panicum turgidium Forsskal		
P. antidotale Forsskal		
Lasiurius scindicus Henrard		
Arrhenatherum elatius (L) Beauv. ex J. & K. Presl	Mortagne, northern France	(Vandenkoornhuyse et al. 2002)

Grassland ecosystems are often characterized by large spatial and temporal fluctuations in resource availability. Under these conditions, the development of transient symbioses is an important strategy to extend plant use of resources. In the North American prairies, for example, the main photosynthetic activity is carried out early in the season by C_3 plants, while C_4 plants are typically active later during

warmer periods (Ode et al. 1980). In addition to their metabolic attributes, both C_3 and C_4 plants usually have root systems that can reach several meters deep and go through layers of soil with different properties (Craine et al. 2003). The combination of plant community composition and climate drives the development of a heterogeneous horizontal and vertical environment in the soil. It is difficult to conceive a single plant or single symbiosis successfully exploiting this scattered distribution of resources during all seasons.

Research efforts have targeted the complex ecological structure of grassland ecosystems (Stanton 1988; Reynolds et al. 2003), but an integrative concept of this ecosystem requires the study of fungal root endophytic symbioses as it varies seasonally and at depth beyond the first 15 or 20 cm. Research on fungal root endophytes has mostly been focussed on the first 10 or 15 cm of the soil for feasibility reasons.

Information gathered from alpine ecosystems could help to understand the relevance of such a perspective in guiding research efforts aimed at understanding the complex interactions between grass roots and fungal root endophytes in grasslands' seasonal dimensions. The information presented by Mullen et al. (1998) is a good example of this situation. They found a high accumulation of N in *Ranunculus adoneus* Gray at the beginning of the growing season, concurrently with the highest concentration of dissolved organic nitrogen and the highest level of root colonization by DSE. The highest accumulation of P in plant was found during the warmer part of the season, coincident with the peak in arbuscule colonization of roots by AM fungi. Such variable multipartite relationships provide benefits for the plant at different times which may explain the investment of organic carbon into different endophytes.

Similarly, the inoculation of *Gnaphalium norvegicum* L, the alpine herb, with a DSE increased plant reproduction fitness by reducing the temperature required for germination, while at higher temperatures AM colonization increased the content of N and biomass accumulation (Ruotsalainen and Kytöviita 2004).

Interactions involving roots and their symbionts can vary under the influence of neighboring plants (Jastrow and Miller 1993), root plastic response (Genney et al. 2000), soil development (Lindahl et al. 2007) or depth per se (Allison et al. 2007). This variation influences the vertical and horizontal distribution of multiple niches for soil microorganisms and their function. For example, the inoculation of the shrub *Calluna vulgaris* (L.) Hull with the ericoid *Hymenosciphus ericae* (Read) Korf & Kernan in a pot study reduced the amount and proportion of roots of the neighboring grass *Nardus stricta* L in the organic layers of the soil (Genney et al. 2000). We observed variations in the distribution of different root endophytes at different depths in the soil profile under single grass species stands (unpublished). In monoculture plots of *Agropyron smithii* Rydb, *Stipa viridula* Trin, *Pascopyrum desertorum* (Fisch. ex Link) Schult, *Elymus junceus* Fisch and *Panicum virgatum* L, we found similar levels of colonization by AM fungi and DSE in roots taken from the first 15 cm of the soil, but the level of colonization by DSE was higher than that of AM fungi at 15–45 cm depth.

The magnitude of day-night and seasonal variations in soil temperature decline with soil depth. Different environment at different depths can certainly be exploited

more effectively by different symbioses in the same plant. The driving force of soil temperature variations along a soil profile in the function of grass root symbioses is unknown, but variable effects of temperature on AM fungi or AM symbioses were reported (see, e.g., Mohammad et al. 1998; Klironomos et al. 2001; Addy et al. 1998; McGonigle and Miller 1999) as was the protective effect of DSE at extreme soil temperatures which is well known (Redman et al. 2002; Rodriguez et al. 2004).

The composition of AM fungal populations varies with soil depth (Oehl et al. 2005), and different AM fungi have different extramatrical development (Hart and Reader 2002). Since nitrogen and water are the most limiting factors for plant growth in many grassland ecosystems, it makes sense that the most successful plants are those exploiting the highest number of transient symbioses involved with nitrogen acquisition or water relations in multiple niches of the soil environment.

Organic N is the most important source of N not only in nonfertilized grasslands (Bardgett et al. 2003) but also in many agricultural systems (Lipson and Näsholm 2001). Several DSE can decompose organic matter and liberate plant available N (Mandyam and Jumpponen 2005) and perhaps promote the absorption of intact amino acids by plants (Miller and Bowman 2002). These fungi can exhibit a surprisingly high metabolic activity (Schmidt et al., unpublished) and high colonization of roots (Mullen et al. 1998) at low temperatures. Arbuscular mycorrhizal fungi can mobilize inorganic forms of N (Mader et al. 2000), but seem to be affected by low soil temperatures (Klironomos et al. 2001; Heinemeyer and Fitter 2004; Mohammad et al. 1998) to a larger extent than DSE.

On the other hand, vertical water distribution is not uniform in xeric environments. Both, DSE (Barrow 2003) and AM fungi (Allen et al. 1981) could improve grass water use efficiency by improving the soil to root hydraulic conductance.

7 Symbiotic Fungi Biotechnology

The development and use of various biotechnologies has become a necessity to reduce farmers' reliance on agrochemical inputs. Mineral fertilizers, while increasing yield, have contributed to environmental problems related to leakage of nutrients out of the soil to the environment, such as nitrous oxide emission to the atmosphere and eutrophication of water bodies. It is now well-recognized that microbial inoculants containing beneficial organisms such as AM fungi offer a potential to enhance nutrient use efficiency of crop plants and help preserve the quality of the environment (Saito and Marumoto 2002). These inoculants can maximize crop yields and quality while minimizing applications of chemical fertilizers and pesticides that can be harmful to people and the environment. Microbial inoculants are carrier-based preparations containing beneficial microorganisms in a viable state that are intended for seed or soil application, and designed to help plant growth by increasing the number and biological activity of beneficial

microorganisms in the root environment (Subba Rao 1993). These inoculants may include bioactive molecules, sometimes called signal molecules, to improve symbiotic formation and function (Mabood et al. 2006). Therefore, for agronomic purpose, inoculation of plants with selected microorganisms at a much higher concentration than those normally found in soil, or stimulation of native beneficial microorganisms is necessary to take advantages of their beneficial properties for plant yield enhancement (Valverde et al. 2006).

In addition to numerous species of bacteria, a few nonsymbiotic fungal species, including *Aspergillus awamori, Penicillium digitatum*, and *Trichoderma* sp., are used as inoculants for crop production purposes. These are used as phosphate solubilizing agents to increase the availability of P in soils (Wani and Lee 2002). AM fungi, the best-known group of symbiotic fungal endophytes, are also used as inoculants. The mycorrhizal symbiosis provides many benefits to plants such as increased absorption of nutrients and water. It likewise serves as a biological control agent against root infection and can improve soil properties. Because all of these beneficial effects, AM fungi were also evaluated to produce different AM-based inoculants.

Evidence of the AM inoculants benefits to agricultural accumulates. The obligate biotrophic nature of AM fungi inevitably raises the cost for inoculum production relative to that of culturable organisms (Saito and Marumoto 2002), but with escalating prices of mineral fertilizers, AM inoculants are becoming a viable option even for agronomic crop production, in Canada. In addition, the effectiveness of AM fungal inoculation is affected by various environmental and biological factors, especially the P availability in soil and the inoculum potential of indigenous AM fungi (Gianinazzi et al. 1990). Because of these problems, the attention also turned to managing existing mycorrhizal fungal populations, such as by minimizing soil disturbance, reducing fallow periods, the application of chemical stimulants of the symbiosis, and the proper use of pesticides (Koide and Mosse 2004; Plenchette et al. 2005).

8 Conclusion

Fungal root endophytes should be better exploited in agriculture. This is a challenge, given the complexity of the living soil. It has traditionally been easier to ignore the mechanisms of the soil system and support the agricultural industry with large amounts of inefficiently used agrochemical inputs. The pressure on agricultural land to produce food for the increasing human population, and more recently to produce biofuels, is sharply rising. New solutions are required to resolve the problems of soil, water and air quality degradation related to crop production. Our living world is characterized by intricate and complex relationships between its components. The selection of less disturbing cropping practices and protecting AM networks in soil will help reduce the environmental impacts of crop production.

References

Abiven S, Menasseri S, Angers DA, Leterme P (2007) Dynamics of aggregate stability and biological binding agents during decomposition of organic materials. Eur J Soil Sci 58:239–247

Addy HD, Boswell EP, Koide RT (1998) Low temperature acclimation and freezing resistance of extraradical VA mycorrhizal hyphae. Mycol Res 102:582–586

Albuquerque JA, Mafra AL, Fontoura SMV, Bayer C, dos Passos JFM (2005) Avaliaçao de sistemas de preparo e calagem em um latossolo bruno aluminico. R Bras Ci Solo 29:963–975

Al-Karaki G N and Clark R B (1999) Mycorrhizal influence on protein and lipid of durum wheat grown at different soil phosphorus levels. Mycorrhiza 9:97

Allen EB, West NE (1993) Nontarget effects of the herbicide tebuthiuron on mycorrhizal fungi in sagebrush semidesert. Mycorrhiza 3:75–78

Allen MF, Smith WK, Moore TS Jr, Christensen M (1981) Comparative water relations and photosynthesis of mycorrhizal and non-mycorrhizal *Bouteloua gracilis* H. B. K. Lag Ex Steud. New Phytol 88:683–693

Allison VJ, Miller RM, Jastrow JD, Matamala R, Zak DR (2005) Changes in soil microbial community structure in a tallgrass prairie chronosequence. Soil Sci Soc Am J 69:1412–1421

Allison VJ, Yermakov Z, Miller RM, Jastrow JD, Matamala R (2007) Using landscape and depth gradients to decouple the impact of correlated environmental variables on soil microbial community composition. Soil Biol Biochem 39:505–516

Antunes PM, Deaville D, Goss MJ (2006a) Effect of two AMF life strategies on the tripartite symbiosis with *Bradyrhizobium japonicum* and soybean. Mycorrhiza 16:167–173

Antunes PM, Rajcan I, Goss MJ (2006b) Specific flavonoids as interconnecting signals in the tripartite symbiosis formed by arbuscular mycorrhizal fungi, *Bradyrhizobium japonicum* (Kirchner) Jordan and soybean (*Glycine max* (L.) Merr.). Soil Biol Biochem 38:533–543

Azcon R, Gomez-Ortega M, Barea JM (1982) Comparative effects of foliar-or soil-applied nitrate on vesicular-arbuscular mycorrhizal infection in maize. New Phytol 92:553–559

Baath E, Spokes J (1989) The effect of added nitrogen and phosphorus on mycorrhizal growth response and infection in *Allium schoenoprasum*. Can J Bot 67:3227–3232

Babana AH, Antoun H (2005) Biological system for improving the availability of Tilemsi phosphate rock for wheat (*Triticum aestivum* L.) cultivated in Mali. Nutri Cycl Agroecosyst 72:147–157

Babana AH, Antoun H (2006) Effect of Tilemsi phosphate rock-solubilizing microorganisms on phosphorus uptake and yield of field-grown wheat (*Triticum aestivum* L.) in Mali. Plant Soil 287:51–58

Balser TC, Treseder KK, Ekenler M (2005) Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence. Soil Biol Biochem 37:601–604

Baltruschat H (1985) Auftretan der VA-mycorrhiza in Abhangigkeit verschiedener Herbizidbehandlungen und der Intensitat der stickstoffdungung. VDLUFA-Schriftenreihe. 16:403–412

Bardgett RD, Streeter TC, Bol R (2003) Soil microbes compete effectively with plants for organic-nitrogen inputs to temperate grasslands. Ecology 84:1277–1287

Barrow, J.R (2003) Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. Mycorrhiza 13:239–247

Baumgartner K, Smith RF, Bettiga, L (2005) Weed control and cover crop management affect mycorrhizal colonization of grapevine roots and arbuscular mycorrhizal fungal spore populations in a California vineyard. Mycorrhiza 15:111–119

Bethlenfalvay GL, Ames RN (1987) Comparison of two methods for quantifying extraradical mycelium of vesicular-arbuscular mycorrhizal fungi. Soil Sci Soc Am J 51:834–837

Bethlenfalvay GJ, Mihara KL, Schreiner RP, McDaniel H (1996) Mycorrhizae, biocides, and biocontrol. 1. Herbicide-mycorrhiza interactions in soybean and cocklebur treated with bentazon. Appl Soil Ecol 3:197–204

Bianciotto V, Genre A, Jargeat P, Lumini E, Becard G, Bonfante P (2004) Vertical transmission of endobacteria in the arbuscular mycorrhizal fungus *Gigaspora margarita* through generation of vegetative spores. Appl Environ Microbiol 70:3600–3608

Biermann B, Linderman RG (1983) Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytol 95:97–105

Bittman S, Kowalenko CG, Hunt DE, Forge TA, Wu X (2006) Starter phosphorus and broadcast nutrients on corn with contrasting colonization by mycorrhizae. Agron J 98:394–401

Blanco-Canqui H, Lal R (2004) Mechanisms of carbon sequestration in soil aggregates. Crit Rev Plant Sci 23:481–504

Blanke V, Renker C, Wagner M, Fullner K, Held M, Kuhn AJ, Buscot F (2005) Nitrogen supply affects arbuscular mycorrhizal colonization of *Artemisia vulgaris* in a phosphate-polluted field site. New Phytol 166:981–992

Bolan NS, Robson AD, Barrow NJ (1984) Increasing phosphorus supply can increase the infection of plant roots by vesicular-arbuscular mycorrhizal fungi. Soil Biol Biochem 16:419

Borie F, Rubio R, Rouanet JL, Morales A, Borie G, Rojas C (2006) Effects of tillage systems on soil characteristics, glomalin and mycorrhizal propagules in a Chilean Ultisol. Soil Till Res 88:253–261

Boswell EP, Koide RT, Shumway DL, Addy HD (1998) Winter wheat cover cropping, VA mycorrhizal fungi and maize growth and yield. Agric Ecosyst Environ 67:55–65

Brady NC, Weil RR (2001) The nature and properties of soils, 13 edn. Prentice Hall, Upper Saddle River, N.J.

Bruce A, Smith SE, Tester, M (1994) The development of mycorrhizal infection in cucumber: effects of P supply on root growth, formation of entry points and growth of infection units. New Phytol 127:507–514

Brundrett M (2004) Diversity and classification of mycorrhizal associations. Biol Rev 79:473–495

Castillo C, Rubio R, Jouanet JL, Borie F (2006) Early effects of tillage and crop rotation on arbuscular mycorrhizal fungal propagules in an Ultisol. Biol Fertil Soils 43:83–92

Changjin D, Bin Z (2004) Impact of herbicides on infection and hyphal enzyme activity on AM fungus. Acta Pedol Sinica 41:750–755

Corkidi L, Rowland DL, Johnson NC, Allen EB (2002) Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. Plant Soil 240:299–310

Covacevich F, Echeverria HE, Aguirrezabal LAN (2007) Soil available phosphorus status determines indigenous mycorrhizal colonization of field and glasshouse-grown spring wheat from Argentina. Appl Soil Ecol 35:1–9

Craine JM, Wedin DA, Chapin FS, Reich PB (2003) Relationship between the structure of root systems and resource use for 11 North American grassland plants. Plant Ecol 165:85–100

Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol Ecol 36:203–209

de la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–271

de Souza FA, Declerck S (2003) Mycelium development and architecture, and spore production of *Scutellospora reticulata* in monoxenic culture with Ri T-DNA transformed carrot roots. Mycologia 95:1004–1012

Diedhiou PM, Oerke EC, Dehne HW (2004) Effects of the strobilurin fungicides azoxystrobin and kresoxim-methyl on arbuscular mycorrhiza. J Plant Dis Prot 111:545–556

Douds DD, Glaves L, Janke RR (1995) Effects of tillage and farming system upon populations and distribution of vesicular-arbuscular mycorrhizal fungi. Agric Ecosys Environ 52:111–118

Douds Jr DD, Millner PD (1999) Biodiversity of arbuscular mycorrhizal fungi in agroecosystems. Agric Ecosyst Environ 74:77–93

Duponnois R, Plenchette C, Thioulouse J, Cadet P (2001) The mycorrhizal soil infectivity and arbuscular mycorrhizal fungal spore communities in soils of different aged fallows in Senegal. Appl Soil Ecol 17:239–251

Egerton-Warburton L M, Allen E B (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecol Appl 10:484–496

Eom A H, Wilson G W T, Hartnett D C (2001) Effects of ungulate grazers on arbuscular mycorrhizal symbiosis and fungal community structure in tallgrass prairie. Mycologia 93:233–242

Fester T, Maier W, Strack D (1999) Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria. Mycorrhiza 8:241–246

Fitter AH (1985) Functioning of vesicular-arbuscular mycorrhizas under field conditions. New Phytol 99:257–265

Fracchia S, Mujica MT, Garcia Romera I, Garcia-Garrido JM, Martin J, Ocampo JA, Godeas A (1998) Interactions between Glomus mosseae and arbuscular mycorrhizal sporocarp-associated saprophytic fungi. Plant Soil 200:131–137

Galvez L, Douds DD, Drinkwater LE, Wagoner P (2001) Effect of tillage and farming system upon VAM fungus populations and mycorrhizas and nutrient uptake of maize. Plant Soil 228:299–308

Gamalero E, Trotta A, Massa N, Copetta A, Martinotti MG, Berta G (2004) Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14:185–192

Gavito ME, Miller MH (1998) Changes in mycorrhiza development in maize induced by crop management practices. Plant Soil 198:185–192

Genney DR, Alexander IJ, Hartley SE (2000) Exclusion of grass roots from soil organic layers by *Calluna*: the role of ericoid mycorrhizas. J Exp Bot 51:1117

Gianinazzi S, Trouvelot A, Gianinazzi–Pearson V (1990) Conceptual approaches for the rational use of VA endomycorrhizae in agricultrure: possibilities and limitations. Agric Ecosyst Environ 29:153–161

Girvan MS, Bullimore J, Ball AS, Pretty JN, Osborn AM (2004) Responses of active bacterial and fungal communities in soils under winter wheat to different fertilizer and pesticide regimens. Appl Environ Microbiol 70:2692–2701

Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agric Ecosyst Environ 113:17–35

Guttay AJR (1983) The interaction of fertilizers and vesicular-arbuscualr mycorrhizae in composted plant residues. J Am Soc Hortic Sci 108:222–224

Hamel C, Dalpe Y, Lapierre C, Simard RR, Smith DL (1994) Composition of the vesicular-arbuscular mycorrhizal fungi population in an old meadow as affected by pH, phosphorus and soil disturbance. Agric Ecosyst Environ 49:223–231

Harinikumar KM, Bagyaraj DJ (1989) Effect of cropping sequence, fertilizers and farmyard manure on vesicular-arbuscular mycorrhizal fungi in different crops over three consecutive seasons. Biol Fertil Soils 7:173–175

Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344

Hayman DS (1982) Influence of soils and fertility on activity and survival of vesicular-arbuscular mycorrhizal fungi. Phytopathology 72:1119–1125

Heinemeyer A, Fitter AH (2004) Impact of temperature on the arbuscular mycorrhizal (AM) symbiosis: growth responses of the host plant and its AM fungal partner. J Exp Bot 55:525–534

Helgason T, Daniell T J, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431–431

Hepper CM (1983) The effect of nitrate and phosphate on the vesicular-arbuscular mycorrhizal infection of lettuce. New Phytol 93:389–399

Hijri I, Sykorova Z, Oehl F, Ineichen K, Mader P, Wiemken A, Redecker D (2006) Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol Ecol 15:2277–2289

Jabaji-Hare SH, Kendrick WB (1987) Response of an endomycorrhizal fungus in *Allium porrum* L. to different concentrations of the systemic fungicides, metalaxyl (Ridomil) and fosetyl-Al (Aliette). Soil Biol Biochem 19:95–99

Jakobsen I (1994) Research approaches to study the functioning of vesicular-arbuscular mycorrhizas in the field. Plant Soil 159:141–147
Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234
Jansa J, Mozafar A, Frossard E (2003) Long-distance transport of P and Zn through the hyphae of an arbuscular mycorrhizal fungus in symbiosis with maize. Agronomie 23:481–488
Jastrow JD, Miller RM (1993) Neighbor influences on root morphology and mycorrhizal fungus colonization in tallgrass prairie plants. Ecology 74:561–569
Jayaraman J, Kumar D (1995) VAM fungi-pathogen-fungicide interactions in gram. Indian Phytopath. 48:294–299
Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea J-M (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16
Johnson C, Jarrell W, Menge J (1984) Influence of ammonium:nitrate ratio and solution pH on mycorrhizal infection, growth and nutrient composition of *Chrysanthemum morifolium* var. *Circus*. Plant Soil 77:151–157
Johnson NC (1993) Can fertilization of soil select less mutualistic mycorrhizae? Ecol Appl 4:749–757
Johnson NC, Pfleger FL (1992) Vesicular-arbuscular mycorrhizae and cultural stress. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. ASA, Madison, Wis.I, pp 71–100
Johnson NC, Copeland PJ, Crookston RK, Pfleger FL (1992) Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. Agron J 84:387–390
Johnson NC, Rowland DL, Corkidi L, Egerton-Warburton LM, Allen EB (2003) Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84:1895–1908
Jokela WE (1992) Effect of starter fertilizer on corn silage yields on medium and high fertility soils. J Produc Agric 5:233–137
Joner JE (2000) The effect of long-term fertilization with organic or inorganic fertilizers on mycorrhiza-mediated phosphorus uptake in subterranean clover. Biol Fertil Soils 32:435–440
Jumpponen A (2001) Dark septate endophytes-are they mycorrhizal? Mycorrhiza 11:207–211
Jumpponen A, Johnson LC (2005) Can rDNA analyses of diverse fungal communities in soil and roots detect effects of environmental manipulations-a case study from tallgrass prairie. Mycologia 97:1177–1194
Kabir Z (2005) Tillage or no-tillage: impact on mycorrhizae. Can J Plant Sci 85:23–29
Kabir Z, Koide RT (2000) The effect of dandelion or a cover crop on mycorrhiza inoculum potential, soil aggregation and yield of maize. Agric Ecosyst Environ 78:167–174
Kahiluoto H, Ketoja E, Vestberg M, Saarela I (2001) Promotion of AM utilization through reduced P fertilization 2. Field studies. Plant Soil 231:65
Khan AG (2005) Rote of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Medic Biol 18:355–364
Kjoller R, Rosendahl S (2000) Effects of fungicides on arbuscular mycorrhizal fungi: differential responses in alkaline phosphatase activity of external and internal hyphae. Biol Fertil Soils 31:361–365
Kling M, Jakobsen I (1997) Direct application of carbendazim and propiconazole at field rates to the external mycelium of three arbuscular mycorrhizal fungi species: effect on 32P transport and succinate dehydrogenase activity. Mycorrhiza 7:33–37
Klironomos JN, Hart MM, Gurney JE, Moutoglis P (2001) Interspecific differences in the tolerance of arbuscular mycorrhizal fungi to freezing and drying. Can J Bot 79:1161–1166
Koide RT, Li M (1990) On host regulation of the vesicular-arbuscular mycorrhizal symbiosis. New Phytol 114:59–64
Koide R, Mosse B (2004) A history of research on arbuscular mycorrhiza. Mycorrhiza 14:145–163
Kumar K, Bagyaraj DJ (1999) Mass production of arbuscular mycorrhiza as influenced by some agrochemicals. Proc Nat Acad Sci India 69:61–66

Lekberg Y, Koide RT (2005) Arbuscular mycorrhizal fungi, rhizobia, available soil P and nodulation of groundnut (*Arachis hypogaea*) in Zimbabwe. Agric Ecosyst Environ 110:143–148

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD (2007) Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. New Phytol 173:611–620

LingFei L, Yang A, ZhiWei Z (2005) Seasonality of arbuscular mycorrhizal symbiosis and dark septate endophytes in a grassland site in southwest China. FEMS Microbiol Ecol 54:367–373

Lingfei L, Zhang Y, Zhao Z-W (2007) Arbuscular mycorrhizal colonization and spore density across different land-use types in a hot and arid ecosystem, Southwest China. J Plant Nut Soil Sci 170:419–425

Lipson D, Näsholm T (2001) The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. Oecologia 128:305–316

Liu A, Hamel C, Hamilton R, Smith D (2000) Mycorrhizae formation and nutrient uptake of new corn (*Zea mays* L.) hybrids with extreme canopy and leaf architecture as influenced by soil N and P levels. Plant and Soil 221:157

Loveland P, Webb J (2003) Is there a critical level of organic matter in the agricultural soils of temperate regions: a review. Soil Till Res 70:1–18

Lovera M, Cuenca G (2007) Diversity of arbuscular mycorrhizal fungi (AMF) and mycorrhizal potential of the soil from a natural and a disturbed savannah from La Gran Sabana, Venezuela. Interciencia 32:108–114

Lu S, Braunberger P, Miller M (1994) Response of vesicular-arbuscular mycorrhizas of maize to various rates of P addition to different rooting zones. Plant Soil 158:119

Mabood F, Gray EJ, Lee KD, Supanjani, Smith DL. (2006). Exploiting inter-organismal chemical communication for improved inoculants. Can J Plant Sci 86:951–966

Mader P, Vierheilig H, Streitwolf-Engel R, Boller T, Frey B, Christie P, Wiemken A (2000) Transport of 15N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi. New Phytol 146:155–161

Malty JS, Siqueira JO, Moreira FMS (2006) Efeitos do glifosato sobre microrganismos simbiotroficos de soja, em meio de cultura e casa de vergetacao. Pessq Agropec Bras Brasilia 41:285–291

Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Studies in Mycology 53:173–189

Manlay RJ, Feller C, Swift MJ (2007) Historical evolution of soil organic matter concepts and their relationships with the fertility and sustainability of cropping systems. Agric Ecosys Environ 119:217–233

Marin M, Ybarra M, Fe A, Garcia-Ferriz L (2002) Effect of arbuscular mycorrhizal fungi and pesticides on Cynara cardunculus growth. Agric Food Sci Finland 11:245–251

Martensson AM, Carlgren K (1994) Impact of phosphorus fertilization on VAM diaspores in two Swedish long-term field experiment. Agric Ecosys Environ 47:327–334

Martinez A, Obertello M, Pardo A, Ocampo JA, Godeas A (2004) Interactions between Trichoderma pseudokoningii strains and the arbuscular mycorrhizal fungi Glomus mosseae and Gigaspora rosea. Mycorrhiza 14:79–84

McAllister CB, Garcia Romera I, Godeas A, Ocampo JA (1994) Interactions between Trichoderma koningii, Fusarium solani and Glomus mosseae: effects on plant growth, arbuscular mycorrhizas and the saprophyte inoculants. Soil Biol Biochem 26:1363–1367

McGonigle TP, Miller MH (1999) Winter survival of extraradical hyphae and spores of arbuscular mycorrhizal fungi in the field. Appl Soil Ecol 12:41–50

Menéndez AB, Scervino JM, Godeas AM (2001) Arbuscular mycorrhizal populations associated with natural and cultivated vegetation on a site of Buenos Aires province, Argentina. Biol Fertil Soils 33:373–381

Menge JA, Johnson ELV, Minassian V (1979) Effect of heat treatment and three pesticides on the growth and reproduction of the mycorrhizal fungus Glomus fasciculatus. New Phytol 82:473–480

Merryweather JW, Fitter AH (1998) Patterns of arbuscular mycorrhiza colonisation of the roots of *Hyacinthoides non-scripta* after disruption of soil mycelium. Mycorrhiza 8:87–91
Miller AE, Bowman WD (2002) Variation in nitrogen-15 natural abundance and nitrogen uptake traits among co-occurring alpine species: do species partition by nitrogen form? Oecologia 130:609–616
Miller MH (2000) Arbuscular mycorrhizae and the phosphorus nutrition of maize: a review of Guelph studies. Can J Plant Sci 80:47–52
Miller MH, McGonigle TP, Addy HD (1995) Functional ecology of vesicular arbuscular mycorrhizas as influenced by phosphate fertilization and tillage in an agricultural ecosystem. Crit Rev Biotechnol 15:241–255
Miller RM, Miller SP, Jastrow JD, Rivetta CB (2002) Mycorrhizal mediated feedbacks influence net carbon gain and nutrient uptake in *Andropogon gerardii*. New Phytol 155:149–162
Miranda JCC, Harris PJ (1994) Effects of soil phosphorus on spore germination and hyphal growth of arbuscular mycorrhizal fungi. New Phytol 128:103–108
Mohammad MJ, Pan WL, Kennedy AC (1998) Seasonal mycorrhizal colonization of winter wheat and its effect on wheat growth under dryland field conditions. Mycorrhiza 8:139–144
Mosse B (1973) Plant growth responses to vesicular-arbuscular mycorrhiza. New Phytol 72:127–136
Mujica MT, Fracchia S, Menendez A, Ocampo JA, Godeas A (1998) Influence of chlorsulfuron herbicide on arbuscular mycorrhizas and plant growth of Glycine max intercropped with the weeds *Brassica campestris*. Proceedings of the 2nd International Conference on Mycorrhiza, Uppsala, Sweden, pp 5–10
Mullen RB, Schmidt SK, Jaeger CH (1998) Nitrogen uptake during snowmelt by the snow buttercup, *Ranunculus adoneus*. Arct Alp Res 30:121–125
Muthukumar T, Senthilkumar M, Rajangam M, Udaiyan K (2006) Arbuscular mycorrhizal morphology and dark septate fungal associations in medicinal and aromatic plants of Western Ghats, Southern India. Mycorrhiza 17:11–24
Nagahashi G, Douds Jr DD (2000) Partial seperation of root exudates components and their effects upon the growth of germinated spores of AM fungi. Mycol Res 104:1453–1464
Nagahashi G, Douds Jr DD, Abney GD (1996) Phosphorus amendment inhibits hyphal branching of the VAM fungus *Gigaspora margarita* directly and indirectly through its effect on root exudation. Mycorrhiza 6:403–408
Nasr AA (1993) The effect of cytokinin and thidiazuron on tomato inoculated with endomycorrhiza. Mycorrhiza 3:179–182
Ode DJ, Tieszen LL, Lerman JC (1980) The seasonal contribution of C3 and C4 plant species to primary production in a mixed prairie. Ecology 61:1304–1311
Oehl F, Sieverding E, Ineichen K, Ris E-A, Boller T, Wiemken A (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 165:273–283
Olsson PA, Baath E, Jakobsen I (1997) Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Appl Environ Microbiol 63:3531–3538
Pattison GS, Warton DL, Misman R, McGee PA (1997) Mycorrhiza 7:155–159
Pinior A, Wyss U, Piché Y, Vierheilig H (1999) Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Bot 77:891–897
Pirozinsky KA, Dalpé Y (1992) The geological history of the Glomaceae with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–36
Plenchette C, Perrin R (1992) Evaluation in the greenhouse of the effects of fungicides on the development of mycorrhiza on leek and wheat. Mycorrhiza 1:59–62
Plenchette C, Clermont-Dauphin C, Meynard JM, Fortin JA (2005) Management of arbuscular mycorrhizal fungi in cropping systems Can J Plant Sci 85:31–40
Rao AV, Tarafdar JC, Sharma SK, Praveen-Kumaraggarwal RK (1995) Influence of cropping systems on soil biochemical properties in an arid rain-fed environment. J Arid Environ 31:237–244

Ratti N, Kumar S, Verma HN, Gautam SP (2001) Improvement in bioavailability of tricalcium phosphate *to Cymbopogon martinii* var. *motia* by rhizobacteria, AMF and *Azospirillum* inoculation. Microbiol Res 156:145–149

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance generated by plant/fungal symbiosis. Science 298:1581

Rejon A, Garcia-Romera I, Ocampo JA, Bethlenfalvay GJ (1997) Mycorrhizal fungi influence competition in a wheat-ryegrass association treated with the herbicide diclofop. Appl Soil Ecol 7:51–57

Reynolds HL, Packer A, Bever JD, Clay K (2003) Grassroots ecology: plant-microbe-soil interactions as drivers of plant community structure and dynamics. Ecology 84:2281–2291

Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitigation Adaptation Strategies Global Change 9:261–272

Ruotsalainen AL, Kytöviita MM (2004) Mycorrhiza does not alter low temperature impact on *Gnaphalium norvegicum*. Oecologia 140:226–233

Ryan MH, Chilvers GA, Dumaresq DC (1994) Colonisation of wheat by VA-mycorrhizal fungi was found to be higher on a farm managed in an organic manner than on a conventional neighbour. Plant Soil 160:33–40

Ryan MH, Small DR, Ash JE (2000) Phosphorus controls the level of colonisation by arbuscular mycorrhizal fungi in conventional and biodynamic irrigated dairy pastures. Aust J Exp Agric 40:663–670

Saito M, Marumoto T (2002) Inoculation with arbuscular mycorrhizal fungi: the status quo in Japan and the future prospects. Plant Soil 244:273–279

Salem SF, Dobolyi C, Helyes L, Pck Z, Dimcny J (2003) Side-effect of benomyl and captan on arbuscular mycorrhizal formation in tomato plant. ISHS Acta Hortic 613:243-246.

Sattelmacher B, Reinhard S, Pomikalako A (1991) Differences in mycorrhizal colonization of rye (Secale cereale L.) grown in conventional or organic (biological-dynamic) farming systems. J Agron Crop Sci 167:350–355

Schalamuk S, Velazquez S, Chidichimo H, Cabello M (2006) Fungal spore diversity of arbuscular mycorrhizal fungi associated with spring wheat: effects of tillage. Mycologia 98:16–22

Schreiner RP, Bethlenfalvay GJ (1997) Plant and soil response to single and mixed species of arbuscular mycorrhizal fungi under fungicide stress. Appl Soil Ecol 7:93–102

Schwab SM, Johnson ELV, Menge JA (1982) Influence of simazine on formation of vesicular-arbuscular mycorrhizae in Chenopodium quinona Willd. Plant Soil 64:283–287

Schweiger PF, Jakobsen I (1998) Dose-response relationships between four pesticides and phosphorus uptake by hyphae of arbuscular mycorrhizas. Soil Biol Biochem 30:1415–1422

Sieverding E (1991) Vesicular-arbuscular management in tropical agrosystems. Deutsche Gesellschaft für Technische Zusammenarbeit, Eschborn, Federal Republic of Germany

Sieverding E, Leihner DE (1984) Effect of herbicides on population Dynamics of VA-mycorrhiza with cassava. Angew Botanik 58:283–294.

Siquiera JO, Safir GR, Nair MG (1991) VA-mycorrhizae and mycorrhiza stimulating isoflavanoid compounds reduce plant herbicide injury. Plant Soil 134:233–242

Six J, Bossuyt H, Degryze S, Denef K (2004) A history of research on the link between (micro)aggregates, soil biota, and soil organic matter dynamics. Soil Till Res 79:7–31

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, New York

Srinath J, Bagyaraj D, Satyanarayana B (2003) Enhanced growth and nutrition of micropropagated Ficus benjamina to Glomus mosseae co-inoculated with Trichoderma harzianum and Bacillus coagulans. World J Microbiol Biotechnol 19:69–72

Stanton NL (1988) The underground in grasslands. Annu Rev Ecol Syst 19:573–589

Subba Rao NS (1993) Biofertilizers in agriculture and forestry. IBH, New Delhi

Sukarno N, Smith FA, Scott ES, Jones GP, Smith SE (1998) The effect of fungicides on vesicular-arbuscular mycorrhizal symbiosis. III. The influence of VA mycorrhiza on phytotoxic effects following application of fosetyl-Al and phosphonate. New Phytol 139:321–330

Sylvia DM, Neal LH (1990) Nitrogen affects the phosphorus response of VA mycorrhiza. New Phytol 115:303–310

Tawaraya K, Watanabe S, Yoshida E, Wagatsuma T (1995) Effect of onion (*Allium cepa*) root exudates on the hyphal growth of Gigaspora margarita. Mycorrhiza 6:57–59

Tawaraya K, Hashimoto K, Wagatsuma T (1998) Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation by the arbuscular mycorrhizal fungus *Gigaspora margarita*. Mycorrhiza 8:67

Thomson BD, Robson AD, Abbott LK (1986) Effects of phosphorus on the formation of mycorrhizas by Gigaspora calospora and Glomus fasciculatum in relation to root carbohydrates. New Phytol 103:751–765

Thomson BD, Robson AD, Abbott LK (1992) The effect of long-term applications of phosphorus fertilizer on populations of vesicular-arbuscular mycorrhizal fungi in pastures. Aust J Agric Res 43:1131–1142

Toro M, Azco N, Barea J M (1997) Improvement of arbuscular mycorrhiza development by inoculation of soil with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (32P) and nutrient cycling. Appl Environ Microbiol 63:4408–4412

Trappe JM, Molina R, Castellano, M (1984) Reactions of mycorrhizal fungi and mycorrhiza formation to pesticides. Annu Rev Phytopathol 22:331–359

Valentine AJ, Osborne BA, Mitchell DT (2001) Interactions between phosphorus supply and total nutrient availability on mycorrhizal colonization, growth and photosynthesis of cucumber. Sci Hortic 88:177–189

Valverde A, Burgos A, Fiscella T, Rivas R, Velázquez E, Rodríguez-Barrueco C, Cervantes E, Chamber M, Igual J-M (2006) Differential effects of coinoculations with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacterium) and *Mesorhizobium ciceri* C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions. Plant Soil 287:43–50

Vandenkoornhuyse P, Baldauf SL, Leyval C, Straczek J, Young JPW (2002) Extensive fungal diversity in plant roots. Science 295:2051

Vandenkoornhuyse P, Ridgway KP, Watson IJ, Fitter AH, Young JPW (2003) Co-existing grass species have distinctive arbuscular mycorrhizal communities. Mol Ecol 12:3085–3095

Vivas A, Marulanda A, Gomez M, Barea JM, Azcon R (2003) Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by Bacillus thuringiensis inoculation under two phosphorus levels. Soil Biol Biochem 35:987–996

von Alten H, Lindemann A, Schonbeck F (1993) Stimulation of vesicular-arbuscular mycorrhiza by fungicides or rhizosphere bacteria. Mycorrhiza 2:167–173

Vyas SC, Vyas S (2000) Effect of agrochemicals on mycorrhizae. In: Mukerji KG, Chamola BP, Singh J (eds) Mycorrhizal biology. Kluwer, New York, pp 289–327

Wan MT, Rahe JE (1998) Impact of azadirachtin on *Glomus intraradices* and vesicular-arbuscular mycorrhiza in root inducing transferred DNA transformed roots of *Daucus carota*. Environ Toxicol Chem 17:2041–2050

Wani SP, Lee KK. (2002). Biofertilizers for sustainable crop production. In: Kannaiyan S (ed) Biotechnology of biofertilizers. Narosa, New Delhi, pp 50–64

Wilberforce EM, Boddy L, Griffiths R, Griffith GW (2003) Agricultural management affects communities of culturable root-endophytic fungi in temperate grasslands. Soil Biol Biochem 35:1143–1154

Xavier LJC, Germida JJ (1997) Growth response of lentil and wheat to *Glomus clarum* NT4 over a range of P levels in a Saskatchewan soil containing indigenous AM fungi. Mycorrhiza 7:3–8

Macroecology of Microbes – Biogeography of the Glomeromycota

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1 Introduction

1.1 Why Study Glomeromycotan Biogeography?

Arbuscular mycorrhizal (AM) fungi are among the most abundant soil microorganisms, associating with 95% of plant families and occurring on all continents of the globe (Smith and Read 1997; Trappe 1987; Read 1991). All AM fungi are members of the newly created phylum Glomeromycota (Schüβler 2001). They inhabit most latitudes and terrestrial ecosystems worldwide, including both natural and human impacted systems. Despite their prevalence in the environment and importance to plant productivity, much remains unknown about patterns of diversity and the biogeography of Glomeromycotan fungi. Biogeography is defined as the study of the geographic distributions of organisms and the mechanisms that drive these distributions. Traditionally, AM fungal diversity was thought to be locally high and globally low; up to 20 species can associate with an individual plant, but less than 250 species have been described worldwide (Morton et al. 1995; Bever et al. 2001). Furthermore, international germ collections have been established in North America and Europe where researchers from around the world can send soil samples to be cultured and archived. According to these collections, many communities from around the globe appear similar, with the same morphospecies such as *Glomus intraradices* seeming to occur globally (Morton and Bentivenga 1994). Over the years, the number of morphospecies in international germ collections has remained low while the number of accessions has increased, indicating low global biodiversity for AM fungi. Furthermore, many taxonomic species such as *Glomus intraradices* and *Glomus mosseae* have been observed in a variety of geographic locations in drastically different environmental conditions. Together, these observations have contributed to the notion that AM fungal species have global distributions. However, critics claim that much of the biogeographical inferences currently made

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about AM fungi are based on information gained from biased sampling and variable methods (Fitter 2005; Johnson and Wedin 1997). Indeed, as the number of scientists working with AM fungi increases and novel regions and ecosystems are sampled, new AM fungal taxa as well as novel morphological traits have been discovered (Bever et al. 2001; Kramadibrata et al. 2000). In addition, methods used to determine AM fungal diversity and species composition are shifting from morphological to DNA-based. New techniques, new species concepts, and collaborative research efforts have invigorated studies of AM fungal biogeography. Joining conceptual frameworks and quantitative models with empirical studies will greatly advance our knowledge of Glomeromycotan biogeography.

A better understanding of Glomeromycotan biogeography is important for the following reasons:

1.1.1 Microbial Biogeography is a Frontier in Ecological Research

The biogeography of cryptic and elusive organisms, particularly microbes, is considered an emerging frontier for the advancement of biogeography (Lomolino and Heaney 2004). Much of the research on microbial biogeography can be defined by the single statement "everything is everywhere, but the environment selects", written by Lourens Gerhard Marinus Baas Becking (1934) and inspired by the work of Martinus Beijrinck (1913) (de Wit and Bouvier 2006). The Baas Becking Hypothesis states that microorganisms are not limited in their dispersal capabilities and thus have global distributions; empirical differences in the composition of microbial communities are due to environmental conditions, which promote active (i.e., observable) species and suppress latent species. In other words, all microbial life is distributed worldwide and, in a given location, most microbial species are not observable because of unfavorable environmental conditions. Baas Becking developed these concepts after decades of work comparing algae, brine shrimp, and bacterial communities among salt lakes in California and all over the world (Baas Becking 1930; Baas Becking 1934).

Although the Baas Becking Hypothesis has influenced microbial ecology for nearly 100 years, new technologies have led to a recent surge in microbial biogeography research. Currently, DNA-based techniques are advancing studies of microbial communities in natural systems, and it is now accepted that traditional morphological and culturing techniques have grossly underestimated microbial diversity (Fry 1990; Torsvik et al. 1990; Tiedje 1995). A recent review of microbial studies that primarily used DNA-based techniques showed that environmental conditions do alter microbial community composition on a variety of spatial and temporal scales, but that microorganisms may not have global distributions (Martiny et al. 2006). This suggests that microbial biogeographical patterns may be more complex than the Baas Becking Hypothesis initially predicted; everything is not everywhere *and* the environment selects. Researchers are attempting to illuminate other patterns in microbial biogeography, such as distance–decay relationships, taxa–area relationships, and local:global taxa richness ratios (Green and Bohannan

2006; Green et al. 2004; Horner-Devine et al. 2004). Mycorrhizologists could learn a great deal from recent insights in the biogeography of prokaryotes and free-living microbial eukaryotes.

1.1.2 The Wallacean Shortfall

A paucity of knowledge about the biogeography of species is referred to as the "Wallacean Shortfall", after Alfred Russel Wallace's view that the key to understanding and conserving biological diversity is through the knowledge of the geographic distributions of organisms (Lomolino 2004). As discussed above, most of the recent research on microbial biogeography has focused on bacterial, archaeal, and protozoan communities. There is little basic information about the geographic distributions of AM fungi in natural systems. Understanding how the abundances of AM fungal species vary with space will provide insights into the factors that control AM fungal diversity and improve estimates of global biodiversity. Do Glomeromycotan species exhibit random, regular, or clumped distributions at spatial scales ranging from peds to continents? Is AM fungal diversity higher in tropical regions, as is the case for macroorganisms? Is AM fungal diversity higher in more heterogeneous habitats? Is global diversity of the Glomeromycota truly low or is it underestimated due to inadequate sampling or resolution of genetic variation? Do anthropogenic activities pose a threat to AM fungal biodiversity? More observational studies of AM fungal species distributions are needed to elucidate answers to such basic, but unexplored questions.

1.1.3 Unique Properties of AM Fungi could Contribute to Biogeographic Patterns

There are reasons to believe that AM fungal biogeography should be different from that of the free-living or even other symbiotic microbes. First, AM fungi are obligate biotrophs that are tightly linked with living host plants. Because of this close relationship with plants, their biogeographical patterns could mirror those of highly mycotrophic plants or plant families. Furthermore, AM fungi associate with members of every major plant clade (Helgason and Fitter 2005). This makes them different from other symbiotic root microbes, such as nodulating rhizobia, which only associate with plants in the Fabaceae family. The strong dependence on plant hosts and general ability to colonize a wide variety of plant lineages makes AM fungi an interesting case study in microbial biogeography.

And second, the ancient Ordovician origins of AM fungi could contribute to unique biogeographical patterns created by historical processes. The earliest fossil records of AM fungi are dated prior to the breakup of Pangaea and formation of separate supercontinents and continents (Redecker et al. 2000; Simon 1993). In fact, mycorrhizal associations were thought to be integral to the establishment and diversification of land plants (Helgason and Fitter 2005; Pirozynski and Malloch

1975). Historical biogeographic factors such as continental drift could have contributed to present-day distributions of AM fungal species. In the subdiscipline of historical biogeography, the theory of continental drift and plate tectonics can be combined with phylogenetic data to make inferences about the history of speciation and biotic assembly within and among geographic regions (Riddle and Funk 2004). Applying these techniques to the study of Glomeromycotan biogeography could give insight into AM fungal phylogeography and diversification history. Therefore, a better understanding of Glomeromycotan biogeography could also provide insights into AM fungal microevolutionary and macroevolutionary processes.

1.1.4 Soil Conservation Requires a Better Understanding of AM Fungal Biogeography

The importance of conserving soil and the ecosystem functions and services it provides is increasingly recognized, as soil loss, degradation, and contamination are more prevalent in natural and managed systems (Daily et al. 1997). Mycorrhizal fungi provide many important ecosystem functions and services at multiple scales: they influence resource acquisition in individual plants, productivity and diversity in plant communities, above- and belowground herbivore interactions, nutrient cycling, soil stability, and carbon sequestration in soils (Newsham et al. 1995; van der Heijden et al. 1998; Gehring and Whitham 2002; Miller and Jastrow 2000; Rillig 2004a; Johnson et al. 2006).

Preserving the functions and services that AM fungi provide in ecosystems requires a better understanding of Glomeromycotan biogeography because individual species and isolates function differently (Hart and Klironomos 2002; Hart and Reader 2002). Different taxa have been shown to provide different growth benefits to plants (Sanders and Fitter 1992; Klironomos 2003). Certain taxonomic groups can also differ morphologically, which could have ramifications for certain ecosystem functions and services. For example, taxa in the Gigasporaceae can produce more dense hyphal networks than those in the Glomeraceae, and these differences may influence soil structure and stability (Miller and Jastrow 2002; Rillig 2004b). In order to conserve the potentially unique ecosystem functions and services that AM fungal species provide, we require a better understanding of their geographic distributions.

Conserving AM fungal diversity in soils across different geographic regions may be important if the natural distributions of certain Glomeromycotan fungi are limited. If individual AM fungal taxa have restricted native distributions then introduction of these soil microbes into new environments could result in unintentional consequences. Production of AM fungal inoculum is a growing industry in North America, with fungi being marketed for agricultural, bioremediation, and restoration industries as well as personal use in home lawns and gardens. Only a few isolates of AM fungi are used universally in these inoculum products. If everything is everywhere, then inoculation with a particular fungal isolate may not be detrimental. However, if the distribution of Glomeromycota is nonrandom,

then inoculation will introduce nonnative and possibly invasive fungal species into new environments. Invasive AM fungi have never been observed in nature. However, this may be the result of inadequate methods to characterize the species composition of AM fungal communities in ecosystems. The potential for an AM fungal species or isolate to become invasive in a foreign introduced environment has never been empirically tested. Schwartz et al. (2006) discuss the possibility of this ecological scenario using examples of ectomycorrhizal fungi that have been documented to invade new environments and cause ecosystem-level alterations (Chapela et al. 2001; Pringle and Vellinga 2006). Because we know that AM fungi influence ecosystem processes in many ways and at many different scales, it is important to determine whether AM fungal inoculum is capable of spreading beyond targeted regions and displacing native AM fungal communities. The goals of this chapter are to:

1) Examine the state of knowledge of Glomeromycotan biogeography and explore the current challenges and benefits of elucidating Glomeromycotan biogeography.
2) Present a conceptual model for the factors that control AM fungal species distributions.
3) Discuss the relevance of spatial scales for the various factors that control AM fungal species distributions.
4) Suggest modeling approaches to address AM fungal biogeographical questions.
5) Generate hypotheses and encourage new research in the area of Glomeromycotan biogeography.

2 Challenges and Benefits of Elucidating Glomeromycotan Biogeography

The scientific discipline of biogeography did not begin with the study of microbes, and therefore the fundamental concept of "species", which is central to the study of geographic ecology, does not seamlessly translate for organisms like AM fungi. The biological species concept, which defines a species according to sexual reproductive isolation, is currently the dominant paradigm for macroorganisms (Mayr 1940). Species concepts for AM fungi, prior to the use of DNA-based techniques, have been predominately morphological. Researchers use asexual spore morphology to distinguish between species and determine AM fungal diversity and community composition (Morton et al. 1995). However, applying the morphospecies concept to AM fungi has several disadvantages. First, characters with which to distinguish species are few and high intraspecific morphological variation is common (Bever and Morton 1999; Bentivenga et al. 1997). Second, spores could reflect legacy communities instead of the species that are actively forming mycorrhizae with plant hosts. And third, not all taxa readily sporulate and therefore lack structures for morphological characterization. It has become evident than many AM fungal species are cryptic in nature and cannot be cultured using current techniques (Clapp

et al. 1995). Furthermore, greenhouse pot cultures do not always reveal the complete fungal community present in the field (Stutz and Morton 1996; Fitter 2005). In a survey in southern Utah, 47 spore morphospecies were found by examining field spores, but only 12 of these species were revealed in greenhouse pot cultures after 2 successive cycles of culturing (Chaudhary 2006). Although the morphospecies concept has a role in AM fungal ecology, it can no longer be the only method used for species identification.

The genetic species concept, where species are grouped by their degree of DNA similarity, has become a dominant paradigm in microbial ecology and could be increasingly useful for Glomeromycotan fungi. An advantage of using the genetic species concept is that DNA used for comparison can be extracted from roots, indicating that it was likely forming active mycorrhizae. Furthermore, this technique does not require spore identification skills, which can be difficult to acquire, time-consuming, and highly variable among researchers. Although DNA analysis can capture more genetic variability than morphological analysis, the proportion of DNA similarity commonly used to distinguish individuals of the same species (e.g., 97%) is often criticized as being chosen arbitrarily.

Perhaps the greatest benefit in nearing a working species concept for AM fungi lies in the combination of molecular and morphological techniques. This approach could contribute to the adoption of either an evolutionary or ecological species concept, which define species by their evolutionary lineages and ecological niches (Simpson 1961; Andersson 1990). According to these species concepts, species share common lineages where lineages evolve separately from others and are under the influence of similar selection pressures. Furthermore, species have a unique role in nature with their own ecological niche in the biotic community. The major challenge to using the evolutionary or ecological species concepts is that they lack simplicity and require a deeper understanding of the biology, evolution, and ecology of AM fungi. However, this understanding will benefit not only studies of biogeography, but also all of Glomeromycotan biology.

2.1 Molecular Techniques Offer an Alternative Approach

Recent methodological advances present mycorrhizologists with new tools to tackle previously unapproachable topics. The development of molecular tools that allow for more accurate in situ species identifications and easier community level analyses have already aided investigations of AM fungal diversity (Wolfe et al. 2007; Lekberg et al. 2007). Developing and applying these techniques is extremely important given the potential limitations and biases of spore and culture based identifications. The PCR-based techniques, such as terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al. 1997), have opened the soil “black box” to more detailed investigations beyond morphological identification colonization quantification and immuno-assays (Horton and Bruns 2001; Johnson et al. 2004; Crawford et al. 2005).

The T-RFLP technique is potentially useful for biogeographical work. T-RFLP produces a community fingerprint for a sample, using PCR with fluorescently-labeled group-specific primers and endonucleases that generate fragments that are read by a laser-sequence analyzer to yield peak profiles. These peak profiles represent the assemblage of species that are present in the sample. However, each peak does not necessarily characterize a single observational taxonomic unit (OTU). This is because of the high potential intra-isolate and intraspecific genetic variation of AM fungi (Bever and Wang 2005; Pawlowska and Taylor 2005). A series of peaks most likely characterize a single OTUs. In order to make the peak to OTU link, a database of peak profiles needs to be generated. This can be done empirically by T-RFLP of single spores or predictively by developing peak profiles through analyzing known sequences for endonuclease cleavage sites. Recently, FitzJohn and Dickie (2006) developed TRAMP-R, a package for the R statistical program that can match unknown T-RFLP profiles with database knowns.

The requirement for an additional identification step makes the technique less advantageous, especially when sequencing technology is rapidly increasing in quality and quantity and decreasing in cost for larger numbers of sequences (Rogers and Venter 2005). Although having greater numbers of sequences would allow for more accurate studies and also greatly aid efforts for greater all-fungi phylogenetic resolution (see Blackwell 2006), the T-RFLP technique will undoubtedly aid AM fungal research as alternative technologies are being developed.

2.2 Resolving AM Fungal Micro- and Macroevolutionary Processes

Past and present evolutionary events and processes affect the spatial distribution of modern taxa. Work on major fossil collections (Redecker et al. 2000) and molecular clock estimates (Simon et al. 1993) have yielded insight into the past evolutionary history of AM fungi. As mentioned in the previous section, advances in molecular techniques and analyses are helping to resolve taxonomic relations both within the Glomeromycota and amongst all fungal taxa. This greater resolution of past fungal evolutionary processes will undoubtedly enhance and direct studies of AM fungal biogeography.

In addition, the current evolutionary processes that create and regulate diversity (i.e. extinction and speciation) play a role in determining AM fungal spatial distributions. Fitness is the determinant of evolutionary success and, therefore, is extremely important to our understanding of biogeography. Estimating fitness for AM fungi can be difficult given their apparent asexuality and clonal life form, but researchers continue to develop new methodological and theoretical approaches (Pringle and Taylor 2002; Pawlowska 2005). Studies of the genomic structure of AM fungi indicate that the genetic diversity of AM fungi is complex and needs further investigation (Sanders 2002; Pawlowska and Taylor 2005; Bever and Wang 2005). However, this is no large exception to the overall difficulties of estimating

fungal population dynamics and fitness. Spore abundance can potentially estimate population size and fitness, but it is problematic to treat spores as individuals in a population when the individual is actually the much larger mycelium that most likely extends well beyond the size of the sampling unit (e.g., a soil core). Furthermore, practical and theoretical problems exist in both measuring the size of AM fungal mycelia and defining the limits of the mycelium when AM fungi have been shown to anastamose (Rosendahl and Stukenbrock 2004; de la Providencia et al. 2005).

Soil is arguably the most complex substrate on earth. The variability of the structure, chemistry, and biological assemblage in soils has yet to be fully realized in the development of a conceptual framework for ecological and evolutionary processes in soils (Crawford et al. 2005). To date, soil ecologists have retrofitted evolutionary ecology theories from aboveground macroorganisms (Martiny et al. 2006). However, this may be insufficient with respect to soil microorganisms, especially fungi. This is at least in part related to their interaction with, and the associated adaptations to, such a complex substrate, but it is also related to inherent differences in their life history and reproductive strategies. Given the high level of spatial and temporal heterogeneity in physical and biological factors, biogeographic studies of soil organisms, especially AM fungi, will benefit greatly from a model-based, exploratory approach.

2.3 A Model for Glomeromycotan Biogeography

Model building is useful for the process of formulating mechanistic hypotheses, identifying gaps in current knowledge, and eventually testing hypotheses with empirical data. Models are particularly powerful when specific predictions and assumptions are made explicit. We formulated a graphical conceptual model of the factors controlling the distribution of AM fungal species for several purposes. First, the model presents the major factors that directly and indirectly influence AM fungal species distributions. Second, the model provides an organized infrastructure in which to discuss the major mechanisms that drive species distributions. The specific prediction of this model is presence or absence of an AM fungal species in a given geographic location. And third, the model highlights hypotheses that have both been empirically tested and those where more research is needed. Mechanisms that influence species distributions vary depending on the scale of the geographic location of interest, a topic that is discussed in greater detail at the end of this chapter.

Both external forces and intrinsic properties of an organism determine the distribution of a species. In our model, factors that influence AM fungal species distributions are separated into three broad categories: abiotic external forces (rectangles), biotic external forces (octagons), and intrinsic properties (ovals) (Fig. 1). It is important to note that although few studies have directly demonstrated the effects of these three factors on species distributions, we have interpreted studies that report changes in

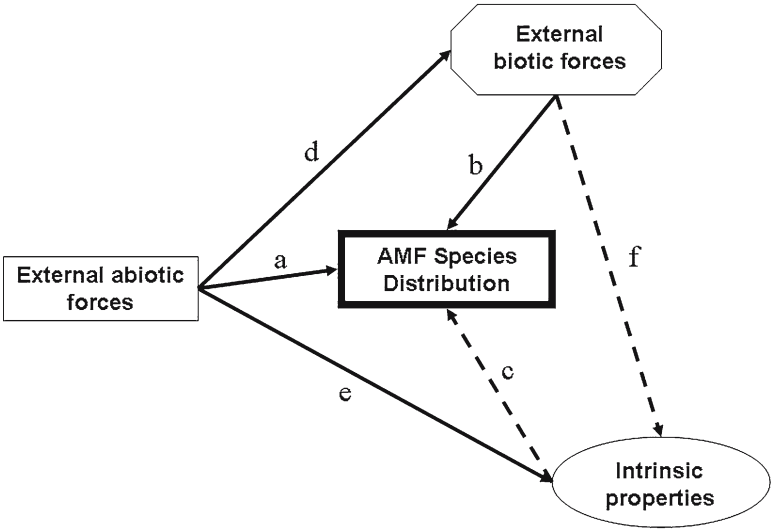

Fig. 1 Factors that influence AM fungal species distributions can be separated into three broad categories: abiotic external forces (*rectangles*), biotic external forces (*octagons*), and intrinsic properties (*ovals*). *Arrows* represent a causal influence that one factor has on either AM fungal species distributions directly (*bold box*) or on another factor. *Solid arrows* represent mechanisms that have support from published research while *dotted arrows* represent possible mechanisms that to our knowledge have never been studied

species abundances or community composition to indicate a potential for environmental effects on species distributions. Furthermore, arrows make no claim as to the strength of each causal influence or sign of the effect (positive or negative). In this way, our model is similar to an a priori model that one would formulate prior to the process of structural equation modeling (Grace 2006). Although the purpose of our model is to organize our understanding of concepts, the direction and relative strength of certain hypothesized relationships could eventually be tested with empirical data.

External abiotic forces, external biotic forces and intrinsic properties of an AM fungal species all directly influence its geographic distribution (Fig. 1). External abiotic forces, such as precipitation or edaphic characteristics, can directly influence the available habitat for a species, which affects an organism's ability to colonize and exist in a given location (Fig. 1, path a). External biotic forces, such as host plant specificity or fungal grazing, can also directly influence the ability of a species to colonize and exist in a particular geographic location (Fig. 1, path b). Paths a and b are solid, indicating that studies have demonstrated how abiotic and biotic external forces influence AM fungal community structure and composition (see discussion below). Intrinsic properties of a species, such as dispersal ability, can also directly influence its ability to colonize a location and therefore its geographic distribution (Fig. 1, path c). Other intrinsic properties such as rate of speciation or extinction can influence whether or not a species will be present in a given geographic location. In our model, path c is dashed to demonstrate the paucity of such autecological research

that addresses how intrinsic biological properties of species influence geographic distributions.

External abiotic forces, external biotic forces and intrinsic properties of species also influence AM fungal geographic distributions indirectly through their interactions with each other (Fig. 1). First, external abiotic forces could indirectly influence AM fungal species distributions by affecting organisms that generate external biotic forces (Fig. 1, path d). For example, precipitation regime could influence the host plant community, which could then influence AM fungal species distributions. Second, external abiotic forces such as climate could also indirectly influence species distributions by influencing intrinsic properties of species such as dispersal ability (Fig. 1, path e). For example, spores of the same AM fungal species may disperse differentially in a dry climate compared to a wet climate. And lastly, external biotic forces could indirectly influence geographic distributions by affecting intrinsic properties of species (Fig. 1, path f). For example, belowground herbivores such as microarthropods could preferentially graze on certain AM fungal species influencing dispersal rates.

In the following sections, we dissect the three main portions of the main model presented in Fig. 1 and discuss in detail the various abiotic and biotic external forces and intrinsic properties that could influence AM fungal species geographic distributions. Because one model with all the parts would be cumbersome to discuss we initially take a modular approach and then conclude with synthesis. Each section is organized in a manner such that direct effects of factors on AM fungal species distributions are discussed first, followed by a discussion of how factors interact to influence AM fungal geographic distributions.

2.4 Abiotic External Forces

2.4.1 Climate

Climate factors, such as precipitation and temperature, could directly influence AM fungal species distributions (Fig. 2, path a). Precipitation can alter soil moisture content, evapotranspiration rates, and plant productivity, all of which can have AM fungal community consequences. In a study of five grasslands in North America that ranged in precipitation from semi-arid to mesic (244–890 mm), Gigasporaceae spores were absent from both semi-arid sites and present in the mesic sites (Johnson et al. 2003). In a comparison of high and low rainfall sites in a wet tropical rainforest in Costa Rica, spores of *Glomus* and *Entrophospora* species were more common in the low rainfall site (Lovelock et al. 2003). In studies where precipitation was constant but soil moisture content varied, AM fungal community differences have also been observed. Across a wide soil hydrological gradient in coastal South Carolina, *Glomus etunicatum, Acaulospora laevis/koskei*, and *Scutellospora heterogama* were found to be intolerant of flood conditions while *Glomus clarum* and *Acaulospora trappei* preferred the wettest sites (Miller and Bever 1999). In a study of California tidal marshes and adjacent upland sites, AM fungal species diversity

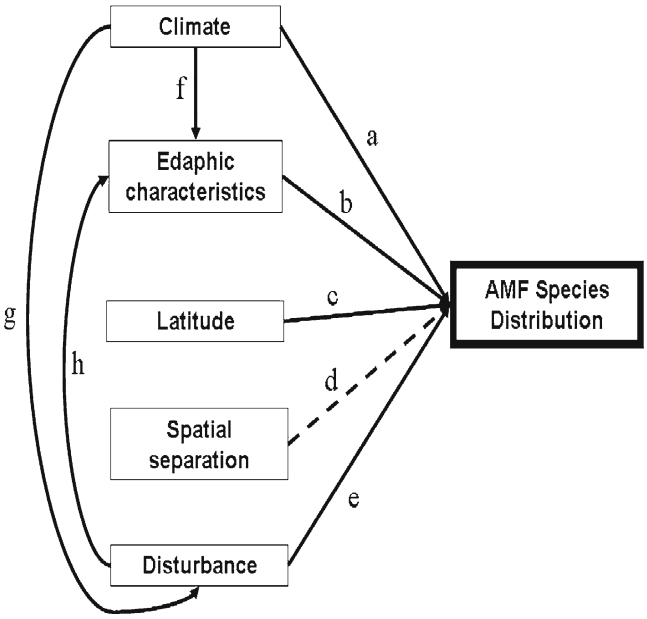

Fig. 2 Abiotic external factors (*rectangles*) that can directly and indirectly influence AMF species distributions *(bold box)*

was found to be similar across soil moisture regimes, but spore abundances differed with soil moisture (Brown and Bledsoe 1996). These field studies suggest that precipitation and soil moisture regimes could influence AM fungal species distributions.

Temperature regimes could also directly influence the geographic distributions of AM fungi as certain species can tolerate – or even thrive in – extreme air and soil temperatures. In greenhouse conditions, warmer air temperatures promoted spore abundance of *Glomus aggregatum, Gigaspora margarita*, and an unknown *Glomus* species (Redman and Johnson, unpublished data). Furthermore, air temperature is not always correlated with soil temperature. In Yellowstone National Park in the western United States, geothermal activity creates a vertical soil temperature gradient such that temperature increases with depth. Researchers have observed AM fungal colonization of plants in soils reaching temperatures of up to 48 °C (Bunn and Zabinski 2003) and extraradical hyphae in soil temperatures too high for roots to exist. Certain AM fungal species may be more tolerant to extreme soil temperatures. Extreme fluctuation of soil temperature, either seasonal or diurnal, is a common stress in nature and a potentially underestimated abiotic factor that drives AM fungal species distributions.

The degree of seasonal or diurnal fluctuation in temperature and precipitation regimes could influence AM fungal species distributions. Seasonal variation in sporulation as well as fungal abundance in roots varies by species (Gemma et al. 1989; Giavanetti 1985). Contrasting and complimentary seasonal phenologies has

been suggested as a possible mechanism that promotes diversity within AM fungal communities. In a North Carolina grassland, *Gigaspora gigantea* sporulated in the cool season while *Acaulospora colossica* sporulated in the warm season (Pringle and Bever 2002). To our knowledge, the influence of diurnal temperature or precipitation fluctuation has never been studied. The habitat heterogeneity hypothesis predicts that regions with low seasonal or diurnal climatic fluctuation should have lower AM fungal alpha or gamma diversity than regions with greater seasonal (e.g., tallgrass prairie) or diurnal (e.g., deserts) climatic fluctuation (Tews et al. 2004). A more heterogeneous habitat could mean more available niche space for species, which could promote persistence of species that disperse into a region. On the other hand, regions with less variable climates may experience less selection events and thus the persistence of a greater number of species. Seasonal and diurnal climate variability should be considered an abiotic external property that influences AM fungal geographic distributions.

2.4.2 Edaphic Characteristics

A substantial body of published literature has shown how edaphic characteristics, such as soil texture and structure, organic matter content, pH, and macronutrient and micronutrient dynamics can influence AM fungal community structure. These studies indicate that edaphic characteristics likely influence AM fungal species distributions in nature (Fig. 2, path b). Soil texture, or particle size distribution, affects many soil properties such as structure, porosity, water holding capacity, and cation exchange capacity. In an experimental garden, *Gigaspora* species preferred sandy soil, while *Entrophospora infrequens* preferred pure sandy loam and *Glomus mosseae* and *Scutellospora calospora* both preferred less sandy soils (Johnson et al. 1992). The observation that *Gigaspora* species preferentially exist in sandy soils is well documented and could be related to the fact that they generally produce large amounts of extraradical hyphae. Communities in sandy soils may shift preferentially towards *Gigaspora* for increased soil stability. Soil organic matter content could also influence AM fungal species distributions although the direct mechanism is unknown.

Most soil organisms subsist in a suitable pH range, but certain AM fungal species have been found to be tolerant to extreme soil pH (reviewed in Abbott and Robson 1991). Furthermore, soil pH affects nutrient availability and plant functioning, which could have indirect effects on AM fungal community structure. In a greenhouse experiment, two AM fungal species were affected differently by liming with $CaCO_3$ (Abbott and Robson 1985). *Glomus fasciculatum* formed mycorrhizae at a pH range of 5.3–7.5 while unidentified *Glomus* isolate WUM16 only formed mycorrhizae at pH 7.5. This evidence suggests that different AM fungal species vary in their pH ranges, which could influence AM community structure. Natural variation in soil acidity in different geographic locations could restrict AM fungal species distributions. Furthermore, human activities that alter soil pH, such as mining, could alter the natural distributions of certain AM fungi.

The influence of soil nutrients, particularly nitrogen and phosphorus, on the AM symbiosis is possibly studied the most by mycorrhizologists. The influence of nitrogen and phosphorus on AM fungal community composition has been examined. Recent work has found that AM fungal community composition shifts in response to anthropogenic nitrogen deposition and nitrogen fertilization (Egerton-Warburton and Allen 2000; Johnson et al. 2003). Furthermore, ambient soil phosphorus influences the response of AM fungi to nitrogen enrichment. Nitrogen-fertilized field plots in phosphorus-rich soil have showed a decrease in *Gigasporaceae* species while nitrogen enrichment of phosphorus-poor soil showed an increase in *Gigasporaceae* species (Egerton-Warburton et al. 2007). These studies indicate that natural variation in nitrogen and phosphorus stoichiometry could influence AM fungal species distributions. Furthermore, anthropogenic increases in N and P deposition will alter the geographic distributions of AM fungi. Many more edaphic characteristics could directly affect AM fungal species distributions, but this area of research has been previously reviewed (Abbott and Robson 1991; Lambert et al. 1980; Porter et al. 1987). Our brevity in this section should not be interpreted to indicate that edaphic properties are not an important control of AM fungal species distributions.

2.4.3 Latitude

Since the early observations of Linnaeus, Humboldt, Darwin, and Wallace that biological diversity is higher in tropical regions compared to temperate regions, ecologists have considered latitude to be a strong force that drives the distributions of many types of organisms on earth. Although there are exceptions to the rule, species richness of macro organisms generally decreases in regions closer to the poles (Hillebrand 2004). Many mechanisms have been proposed to explain the latitudinal diversity gradient including frequency of perturbation, higher productivity, higher environmental heterogeneity, and differences in speciation and extinction rates (Brown 1995). Latitude is correlated with increased surface area, climatic gradients, and solar energy inputs (Rohde 1992). The latitudinal diversity gradient has been referred to as "a pattern in search of a theory" (Rosenzweig 1992). Geographic ecologists who focus their studies on macro organisms are now primarily concerned with demonstrating which mechanisms are responsible for creating higher biodiversity in the tropics (Rohde 1992). Geographic ecologists who focus their studies on microorganisms, in particular soil microbes, must first demonstrate whether or not the latitudinal diversity gradient pattern exists for these organisms. Are soil microbes examples of or exceptions to this widespread ecological pattern?

For the most part, ecologists have yet to determine whether microorganisms exhibit the same latitudinal patterns as macroorganisms. However, some evidence suggests that the community structure of certain microbes changes along latitudinal gradients. For example, communities of gram-negative soil bacteria differed along an 800-km transect, and certain soil fungal communities differed along a southeast to northwest transect in western Canada (Staddon et al. 1998; Morrall 1974). Also,

aerobic anoxygenic phototropic bacterial communities differed along a 20,000-km marine latitudinal transect (Schwalbach and Fuhrman 2005). As studies in microbial biogeography become more prevalent, efforts should be made to not only document geographic variation in community structure, but also the mechanisms that drive biogeographical patterns (Martiny et al. 2006). This is important because studying microbial biogeography could lead to advances in the general field of biogeography. For example, recent studies have investigated speciation rate as a possible mechanism to explain higher biodiversity in the tropics. This hypothesis states that in warmer, more productive environments, metabolic rates and thus mutagenesis rates are higher creating higher rates of evolution and speciation. This hypothesis was supported by an empirical study that showed twice as much nucleotide substitution (ITS region rRNA-encoding DNA) in tropical plants compared to temperate plants (Wright et al. 2006). Furthermore, models that predict genetic divergence and speciation by metabolic rate were confirmed using contemporary and fossil data on planktonic foraminifera (Allen et al. 2006). Certain microorganisms represent a model system in which to empirically test such hypotheses because one can easily manipulate small environments, empirically measure metabolic rates, and observe genetic divergence and evolution.

Some evidence suggests that distributions of AM fungal species vary along latitudinal gradients (Fig. 2, path c), but the mechanisms that contribute to these patterns remain unknown. The issue is further complicated by the fact that AM fungi are obligate symbionts and must always be associated with host plants. Differences in AM fungal communities observed along a latitudinal gradient could be due to host specificity or other environmental variables that change along with latitude. In a latitudinal survey of AM fungal spore communities in coastal dunes along the eastern coast of the United States, plant host was held constant to control for any influence of host specificity (Koske 1987). Along this 355-km transect, AM spore species richness increased and spore community structure shifted with decreasing latitude. *Scutellospora* species such as *S. verrucosa, S. fulgida*, and *S. dipapillosa* dominated the southern half of the transect, but not the northern half. Furthermore, other latitudinal observations of AM fungi have been made such as higher *Sclerocystis* (i.e., *Glomus*), *Acaulospora*, and *Scutellospora* species diversity in the tropics and fewer *Gigaspora* species in northern Europe (Gianinazzi-Pearson and Diem 1982; Herrera-Peraza et al. 2001; Walker 1992). To our knowledge, no studies have been conducted to determine the possible mechanisms that could contribute to the differences in AM fungal community structure along a latitudinal gradient.

More research is needed in many regions of the world with latitudinal gradients of varying sizes to determine the degree to which AM fungal richness and species distributions vary by latitude. These studies are important because they will not only elucidate whether AM fungi follow the latitudinal diversity gradient so prevalent in macroorganisms, but also to increase the amount of species distribution data for tropical regions. Often the task seems daunting because many other factors are confounded with latitude such as changes in climate and host plant communities. However, advances in spatial and statistical modeling present alternatives for controlling environmental conditions, such as measuring the relative contributions of

different environmental factors. Different options and approaches are discussed below in the section on modeling.

2.4.4 Spatial Separation

In general, macroorganisms exhibit strong distance–decay relationships such that community similarity decreases with increasing distance (Nekola and White 1999). This pattern is also interpreted as spatial autocorrelation of community composition or high β–diversity (Magurran 1988). Little is known about the distance–decay relationships of microorgansisms and AM fungi are no exception (Green and Bohannan 2006). Spatial separation can influence AM fungal species distributions (Fig. 2, path d) and can be achieved in a number of ways such as long distances, geographic barriers, or simply a patch of inhospitable soil matrix. Indeed distance-decay relationships vary depending on the scale of study, with certain communities of microbes exhibiting higher β-diversity within continents than between continents (Cho and Tiedje 2000; Franklin and Mills 2003).

Vast geographic separation, such as that found between continents, can create distinct ecological provinces, or regions with biotic communities that reflect the legacy of historical events (de Candolle 1820; Martiny et al. 2006). For example, Australia is often considered a distinct province because it contains many unique animal and plant species that can be attributed to its long isolation from other continents. Tectonic history of a geographic locale can provide insight into length and degree of spatial separation that species from that location have undergone. Advances in plate tectonics have revealed that the configuration of land and sea on the earth is continually changing in time, creating new corridors and barriers to the movement of terrestrial species (Scotese 2004). Furthermore, phylogenetics allows biogeographers to track evolutionary patterns over space and time (Riddle and Funk 2004). The growing subdiscipline of phylogeography aims to understand the geographic distributions of genealogical lineages, bridging ecological and historical aspects of biogeography (Avise 2000; Riddle and Hafner 2004). Microbial biogeographers will benefit from recent advances in molecular techniques that facilitate phylogeographical studies.

The deep evolutionary origins of AM fungi predate the formation of present-day continents; such ancient vicariance events could strongly influence species distributions (Fig. 2, path d). Throughout the Mesozoic, North America and Eurasia made up the supercontinent Laurasia, while South America and Africa together were called Gondwanaland. The continents of North America and Eurasia have been separated by ocean for a shorter period of time than North America and South America. One might expect the AM fungal communities of North America and Europe to be more similar to each other than to the communities of South America or Africa. This might also suggest that global estimates of number of AM fungal species (currently less than 250) could be low since the majority of sampling has occurred in North America and Europe, two ecological provinces that separated early during the break-up of Pangaea. Using new technologies in molecular ecology,

phylogeography, and plate tectonic modeling, great advances could be made to better understand ecological provinces for AM fungi, dispersal capabilities, and the spatial dynamics of AM fungal evolution.

2.4.5 Disturbance History

Disturbances, either natural or human-caused, can directly influence AM fungal species distributions (Fig. 2, path e). A disturbance could disrupt fungal networks, destroying individuals and reducing the distribution of one or many species. The most obvious example, and perhaps the most pressing environmental problem of our time, is the reduction of habitat and associated loss of biodiversity due to human activity (Pimm et al. 1995; Wilcove et al. 1998). On the other hand, certain disturbances can increase available habitat by creating habitat heterogeneity and altering niche availability. Furthermore, in a successional framework, "late-successional" AM fungal communities may diminish while "early-successional" community distributions may increase (Connell and Slayter 1977). Many types of disturbances have the potential to create spatial patterning in species distributions.

Previous studies have shown that natural disturbances can influence the structure and composition of AM fungal communities. Fire can alter AM fungal species distributions depending on the timing, extent, and intensity of the burn (Bentivenga and Hetrick 1991; Eom et al. 1999; Dhillion and Anderson 1993). Forces that initiate primary succession, such as glacial retreat and volcanic flow, create substantial soil disturbance and also influence AM fungal distributions. After the eruption of Mount St. Helens (Washington, U.S.A.) in 1980, AM fungal communities were buried by ash and sterile tephra and were only resurrected in the presence of appropriate animal vectors (Allen 1991; Allen et al. 1987). Forces that initiate secondary succession, such as tropical storms, are generally associated with less soil disturbance and could have less of an impact on AM fungal community structure and composition than primary successional forces (Allen et al. 1998).

A substantial amount of research has shown how human disturbances and land use practices influence AM fungal communities and could therefore impact natural geographic distributions of AM fungi. Anthropogenic disturbances such as mining, grazing, and agriculture, have all been documented to influence AM fungal communities and thus have the potential to create spatial patterning in species distributions (Abbott et al. 1983; Miller 1987; Sieverding 1990; Allen 1991; Johnson and Pfleger 1992; Eom et al. 2001). It is possible that human activities have led to undocumented biodiversity losses in AM fungi, just as they have led to biodiversity losses in animal and plant species.

2.4.6 Abiotic Interactions

Thus far we have discussed how several types of abiotic external factors can directly influence AM fungal communities and species distributions. However,

these abiotic factors can also interact to affect species distributions via a myriad of different mechanisms. First, climate influences edaphic characteristics that in turn affect AMF species distributions (Fig. 2, paths f and b). For example, high mean annual precipitation can leach soils, reducing soil fertility and increasing acidity, both factors that have been shown to influence AM fungal community structure. Second, climate and disturbance can interact to influence AM fungal species distributions (Fig. 2, paths g and e). An example of this process is the differential influence of tillage on AM fungal populations between arid and mesic environments (Jasper et al. 1991). And third, disturbances can change edaphic characteristics that in turn influence AM fungal species distributions (Fig. 2, paths h and b). Disturbances such as fire and intense livestock grazing can change the structure of soil which influences AM fungal communities. Much more research has been done on direct effects of abiotic factors on AM fungal communities than indirect effects or interactions of abiotic factors. Because, in nature, many abiotic factors act in concert, more work is needed on the influence of interactive effects on AM fungal distributions.

2.5 Biotic external forces

2.5.1 Host Plant Community

Glomeromycotan fungi are obligate biotrophs – they cannot live without a plant host (Smith and Read 1997). Because they are symbiotic microorganisms, their biogeographical patterns may be different from that of free-living microbes. Other types of host-associated microbes, from human gut bacteria to leaf parasites, have been observed to exhibit patterns in distribution that are related to their hosts (Falush et al. 2003; Zhang and Blackwell 2002). In a study of nodulating rhizobia in agroecosystems at nine sites across three continents, an ITS phylogenetic analysis yielded 23 different species groups that grouped independently from site, host species, or degree of isolation (Bala et al. 2003). In other words, related rhizobial species associated with different legume species from different sites located on widely separated continents. This study illustrates that obligate, typically-generalist root symbionts can exhibit biogeographical and phylogeographical patterns that are distinct from their plant hosts.

The degree of host-specificity that an AM fungus exhibits will largely determine whether its geographic distribution will be dictated by the host plant community (Fig. 3, path a). Glomeromycotan fungi were largely thought to be generalists, but research has shown that species can exhibit host specificity (Bever et al. 2001; Sanders 2002). In other words, certain AM fungal species preferentially associate with certain plant species. Host-specificity could also occur at the population scale indicating local adaptation (Schultz et al. 2001). It follows that differences in plant community composition coupled with host-specificity could create variation in species distributions of AM fungi. In a meta-analysis of studies that used small subunit

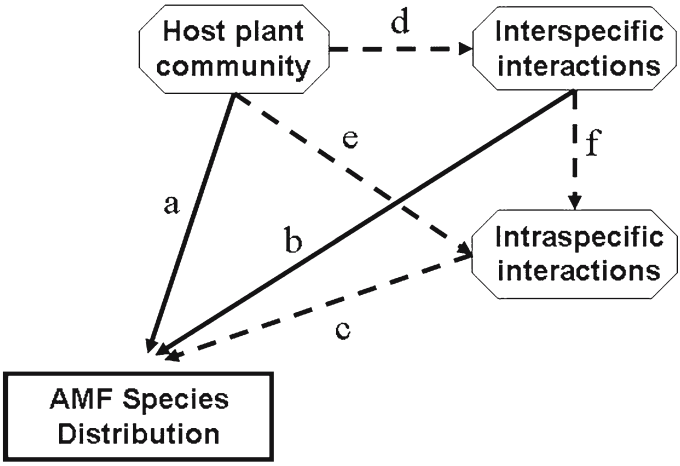

Fig. 3 Biotic factors (*octagons*) that can directly and indirectly influence AMF species distributions *(bold box)*

ribosomal DNA sequences to assess AM fungal species, taxa diversity was compared across sites and different broad vegetation types (Öpik et al. 2006). Although a number of AM fungal taxa had global distributions, 50% of the taxa were recorded from only a single site. Furthermore, AM fungi grouped by vegetation type such that taxa from a tropical forest in Panama were more genetically similar to each other than to those found in grasslands from many regions of the world. However, in a landscape-scale survey of spore communities, where the dominant plant host was always *Artemisia tridentata*, AM fungal species exhibited spatial patterns in distributions regardless of host plant (Allen et al. 1995). Authors attributed these patterns largely to latitude and contemporary environmental conditions. More studies need to be conducted to understand the spatial patterning of AM fungal species as well as the mechanisms that contribute to these patterns. Future studies in AM fungal biogeography should take into consideration the potential for AM fungal species to exhibit strong host-specificity.

Another important issue to consider in the discussion of how plant communities influence AM fungal geographic distributions is the prevalent ecological problem of invasive species. Human-introduced invasive plant species are causing dramatic changes to natural landscapes worldwide and are considered a serious threat to biodiversity (Wilcove et al. 1998). As plant communities shift toward monoculture, the distributions of fungal species could also change due to these invasions. Recent evidence suggests that plant invasions can influence other soil microbes, changing community composition and ecosystem function (Belnap and Phillips 2001; Hawkes et al. 2005; Batten et al. 2006). Some evidence has shown how human-introduced invasive species can influence AM fungal communities (Siguenza et al. 2006a, 2006b). In a recent study, AM fungal T-RFLP profiles of roots from areas dominated by native vegetation were

significantly different from areas dominated by *Centaurea maculosa*, an invasive mycorrhizal forb (Mummey and Rillig 2006). Taxa diversity was also lower in sites dominated by *Centaurea maculosa*. This study suggests that widespread plant invasions have already altered AM fungal communities and, if measures are not taken to control invasive plant species, natural distributions of AM fungal species will continue to be at risk.

2.5.2 Inter- and Intraspecific Interactions

Other organisms besides host plants could directly influence AM fungal species distributions through a variety of different mechanisms such as dispersal, mycophagy, competition, and facilitation. Interspecific interactions could restrict or expand AM fungal species distributions in many ways (Fig. 3, path b). Different types of belowground animals feed on AM fungi, either reducing distributions through consumption or increasing distributions by promoting dispersal (Allen 1991; Gange and Brown 2002). In addition, belowground animals exhibit preferential feeding for certain AM fungal species; depending on the viability of the fungi after passing through the animal's digestive system this interaction could be either beneficial or detrimental for the fungus. Aboveground animals can disperse AM fungal propagules (Gehring et al. 2002), but also compete with the fungi because both share the same plant carbon source (Gehring and Whitham 2002).

In addition to other types of organisms interacting with AM fungi to influence geographic distributions, effects from AM fungi of either different or the same species could influence distributions. Very little is known about how different AM fungal species interact with each other and even less is known about how "individuals" of the same fungal species interact with each other. Studies have shown how AM fungal species grown singly or in a mixed community interact to influence plant productivity and diversity (van der Heijden et al. 1998). However, we know of no studies that have compared the productivity or fitness of fungi grown in a mixed community compared to a fungal monoculture. Furthermore, we know of no studies that have examined the influence of intraspecific interactions on fungal productivity or fitness (Fig. 3, path c). Spatially, positive interspecific or intraspecific interactions could create a clumped distribution while negative interactions might create a regular distribution (as opposed to a random distribution) (Brown et al. 1995). Imagine positive interactions as attractive magnetic forces and negative interactions as two repellent sides of a magnet. These hypotheses suggest that spatial distributions could help elucidate the magnitude and sign of AM fungal species interactions in field conditions.

2.5.3 Biotic Interactions

Biotic factors can influence AM fungal species distributions directly and also indirectly through interactions that the host plant community, interspecific interactions, and intraspecific interactions can have on each other. As with abiotic factors, much

less research has been conducted on how biotic factors interact to indirectly influence AM fungal species distributions compared to direct effects of biotic factors on AM fungi. Nonetheless, these interactions likely exist in nature and we can speculate about several of the possible mechanisms. First, host plant community could influence interspecific interactions, which can then go on to affect species distributions (Fig. 3, paths d and b). In other words, certain interspecific interactions are dependent on the host plant community. For instance, animals that disperse AM fungal propagules may preferentially reside in certain vegetation communities. Second, host plant community could also influence intraspecific interactions, which could then go on to affect AM fungal species distributions (Fig. 3, paths e and c). Although we know of no studies that have examined this process it is feasible that intraspecific interactions could vary depending on the host plant community. A plant's ability to sustain AM fungal individuals could influence the magnitude and sign of intraspecific interactions observed between fungi of the same species. For example, highly dependent plants may sustain more AM fungi, reducing intraspecific competition and encouraging a clumped distribution. On the other hand, facultative hosts may sustain less AM fungi, enhancing intraspecific competition, contributing to a more regular distribution. And third, interspecific interactions could influence intraspecific interactions, going on to affect AM fungal species distributions (Fig. 3, paths f and c). For example, fungal grazers could keep the population size of a particular fungal species low, reducing intraspecific competition and contributing to a clumped spatial distribution. No doubt a myriad of interactions exist among AM fungi and other organisms that could influence the geographic distributions of Glomeromycotan fungi. Most of these interactions remain to be explored.

2.6 Intrinsic properties

2.6.1 Dispersal Ability

Species possess inherent properties that directly and indirectly influence their biogeographical patterns in nature (Fig. 4). The ability of a species to disperse actively through locomotion or passively via a physical or biological agent (e.g., wind, water, animal vectors) can greatly influence its geographic distribution. Microbes have traditionally been thought to have unlimited dispersal capabilities, further contributing to the concept that "everything is everywhere". However, this idea has been recently challenged as body size is not a strong predictor of dispersal capability; many large organisms (e.g., redwood trees) lack long-range dispersal abilities and microbes with active propulsion mechanisms are still unable to cross vast geographic barriers (Martiny et al. 2006). Another type of dispersal, where a species expands its range by moving outward at the boundary of its distribution, is independent of body size and could potentially lead to global distributions of microbes. However, it has been argued that range expansion would be followed by genetic divergence from the

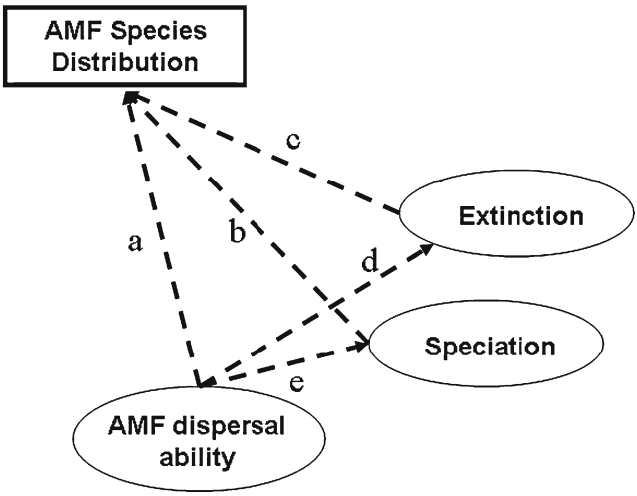

Fig. 4 Intrinsic properties of AM fungal species or isolates (*ovals*) that can directly and indirectly influence their spatial distributions *(bold box)*

source population, creating non-random biogeographical patterns (Martiny et al. 2006). Indeed, just as the dispersal capabilities of macroorganisms are species-specific, microorganisms likely vary in their inherent dispersal abilities as well.

Research has shown that Glomeromycotan fungi are able to disperse in many different ways. Physical forces such as wind and water and biological forces such as animal vectors all act as agents for the passive dispersal of AM fungi. Wind is a substantial dispersal agent in arid and semi-arid environments, moving fungi up to 2 km (Warner et al. 1987). Soil erosion of volcanic, beach, and arid environments can also release fungal propagules (Allen 1991). The influence of aboveground and belowground water movement on AM fungal dispersal is much less understood, but likely plays an important role in dispersal in mesic environments. Animal vectors, from birds to small mammals to nematodes, can disperse spores and hyphal fragments either through ingestion or by the fungi sticking to their bodies (McIlveen and Cole 1976; Ponder 1980; Rabatin and Rhodes 1982; Rothwell and Holt 1978; Allen 1991; Janos et al. 1995; Gange and Brown 2002; Gehring and Whitham 2002). Furthermore, plant hosts could facilitate the belowground range expansion of AM fungi. As hyphal networks expand in the soil matrix they encounter other root zones, initiating new infection points and spreading via "root-to-root" contact (Read et al. 1976; Allen 1991). In certain environmental conditions, hyphal growth can occur at a rate of 30 cm per year. Although research has been conducted to demonstrate several ways in which AM fungi can disperse, much remains to be investigated regarding how AM fungal taxa vary in their dispersal abilities and how this natural variability influences geographic distributions of species.

Although much remains unknown about AM fungal dispersal, inherent differences in dispersal abilities among species likely influence patterns in geographic

distributions (Fig. 4, path a). Fungi in the Glomeromycota also represent an interesting case study in microbial biogeography because the body size of fungal individuals can vary substantially. Fungal individuals comprised of intraradical structures, extraradical hyphal networks and spores can vary in size from millimeters to kilometers, a difference of six orders of magnitude. Spores of certain *Gigaspora* species can be almost 1 mm in diameter while spores of certain *Glomus* species can be smaller than 10 µm. Some morphospecies have highly variable spore sizes, such as *Glomus intraradices* with spores ranging from 40 µm to 190 µm in diameter (Schenk and Perez 1990). Furthermore, portions of the hyphal network of certain AM fungal species are totipotent, able to form new individuals from a single fragment. These observations indicate that body size or spore size alone are poor indicators of dispersal ability.

However, other life history traits indicate that species-specific differences do exist in the dispersal capabilities of AM fungi. Take for example a hypothetical comparison of the dispersal capabilities of two very different AM fungal taxa: Species A and Species B. Species A produces large spores (>500 µm) and extensive extraradical hyphal networks, but does not produce totipotent hyphal fragments. In other words, dispersal in Species A is limited to passive dispersal of spores and range extension via belowground hyphal network expansion. Furthermore, the large spores of Species A may be more likely to disperse via water and animal vectors than wind. Species B on the other hand produces small spores (<100 µm), less extensive extraradical hyphal networks, and totipotent hyphal fragments. Dispersal in Species B can occur via passive dispersal of spores, hyphal fragments and colonized root fragments. Because the spore size of Species B is small it can disperse via wind, water, and animal vectors. Species A could represent a *Gigaspora* species and Species B could represent a *Glomus* species. It is possible that observed differences in the geographic distributions of these two species could be due purely to their inherent differences in dispersal capabilities.

Life history traits of species strongly interact with environmental conditions to determine dispersal capabilities. In an environment where wind is the dominant dispersal agent, Species A might have a more clumped spatial distribution than Species B because its spores are too heavy for passive wind dispersal and it must rely on hyphal network range expansion. However, in an environment where water is the dominant dispersal agent, spores of Species A and Species B would both be dispersed creating similar spatial distributions for both species. Hypotheses regarding how life history strategies influence AM fungal species dispersal and therefore spatial distributions need further testing in a variety of different environments.

2.6.2 Speciation and Extinction

Evolutionary processes contribute to past and present geographic distributions of species (Brown and Gibson 1983). Speciation through the mechanisms of mutation, genetic drift, and natural selection can create genetic differentiation among populations, resulting in new species and a change in the original distribution. Extinction

is achieved through either a localized or complete elimination of a population, resulting in a reduced or eradicated geographic range. In general, speciation rates of microorganisms are believed to be quite high due to high mutation rates, short generation times, high population densities, and mechanisms for genetic recombination such as horizontal gene transfer (Lenski and Travisano 1994; Rainy and Travisano 1998). Microbial biogeographers suggest that high speciation rates coupled with poor dispersal would contribute to non-random biogeographical patterns; indeed certain bacteria have exhibited genetic differentiation and diversification following geographic isolation (Falush et al. 2003). However, microorganisms with high dispersal capabilities could have low speciation rates due to large amounts of gene flow. Extinction rates may also be predictable from population dynamics and dispersal capabilities. For instance, microorganisms with high dispersal capabilities and large populations likely have wide distributions and the ability to avoid complete extinction due to stochastic events. However, microbes with poor dispersal capabilities and small populations may be more prone to extinction (Martiny et al. 2006). Overall, the processes that contribute to speciation and extinction have great potential to create biogeographical patterns in microbial species distributions.

Speciation and extinction events likely influence the geographic distributions of Glomeromycotan species, but it is difficult to hypothesize in what manner or magnitude because a general lack of knowledge exists regarding AM fungal evolutionary processes. AM fungi are coenocytic, meaning their nuclei float freely within an aseptate mycelium. Spores can contain many nuclei; a single *Gigaspora* spore can contain 50,000 nuclei (Clapp et al. 2002). Modes of genetic recombination include mixing of nuclei and anastomosis (Sanders 2002). Evidence suggesting selection exists as well, demonstrating that variable traits of certain species are heritable (Bever and Morton 1999; Bentivenga et al. 1997; Feldman 1998). Because so little is known about the natural diversity and distribution of Glomeromycotan fungi it is difficult to speculate about extinction rates. We are currently in a period of accelerated extinction with an predicted loss of 15% of Central and South American plant species within the next century (Wilson 1988). Consequently, it is conceivable that AM fungal species are being lost before they have been discovered.

2.6.3 Interactions between Intrinsic Properties

Intrinsic properties of species influence AM fungal species distributions both directly and indirectly through their interactions with each other. Dispersal capabilities can interact with extinction and speciation rates to influence geographic distributions of species. For instance, species with high dispersal (e.g., Species B above) may be less likely to experience extinction events and thus have large geographic distributions (Fig. 4, paths d and c). On the other hand, species with low dispersal (e.g., Species A) may not be able to traverse geographic barriers, creating isolation and contributing to allopatric speciation and changing species distributions (Fig. 4, paths e and b). Such indirect effects that intrinsic properties could have on geographic distributions in nature could be substantial. In Fig. 4, the majority of the

pathways are dashed, indicating a serious lack of understanding of AM fungal biology, autecology, and evolutionary processes, knowledge essential to understanding the mechanisms behind Glomeromycotan biogeography.

2.7 Spatial Scaling

Observed geographic patterns greatly depend on the scale of the study and variations in distributions or spatial patterning are only meaningful if the scale of measurement is adequately defined. The models presented in this chapter highlight the external forces and intrinsic properties that influence the geographic distributions of Glomeromycotan fungal species. Each factor that can create variation in species distributions is relevant at a variety of different scales (Fig. 5). Also, distributions of soil microbes likely do not remain constant across scales. A species that exhibits a patchy distribution at the field scale may exhibit a random distribution in the core scale. Different AM fungal species can have distinct distributions as a result of life-history traits, environmental heterogeneity, host distributions or historical factors such as propagule dispersal or small-scale disturbances (Hart and Reader 2002; Ettema and Wardle 2002).

We identify seven different spatial scales that are relevant to the study of AM fungal species distributions (Fig. 6). No strict dimensions of each scale are given because the scales exist along a spatial continuum with each larger scale comprising many of the previous smaller scales. The ped, a single soil aggregate, is among the smallest natural units of soil (Singer and Munns 2002). The next largest spatial scale is the core, which is distinguished from the ped scale because a core contains

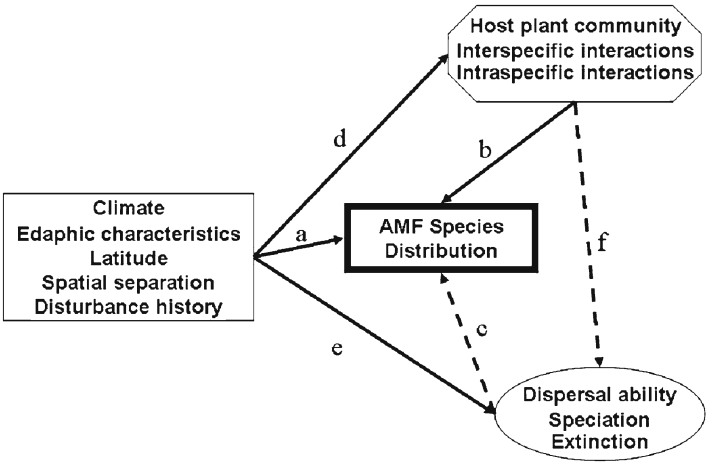

Fig. 5 Culmination of the factors that influence AM fungal species distributions: abiotic external forces (*rectangles*), biotic external forces (*octagons*), and intrinsic properties *(ovals)*

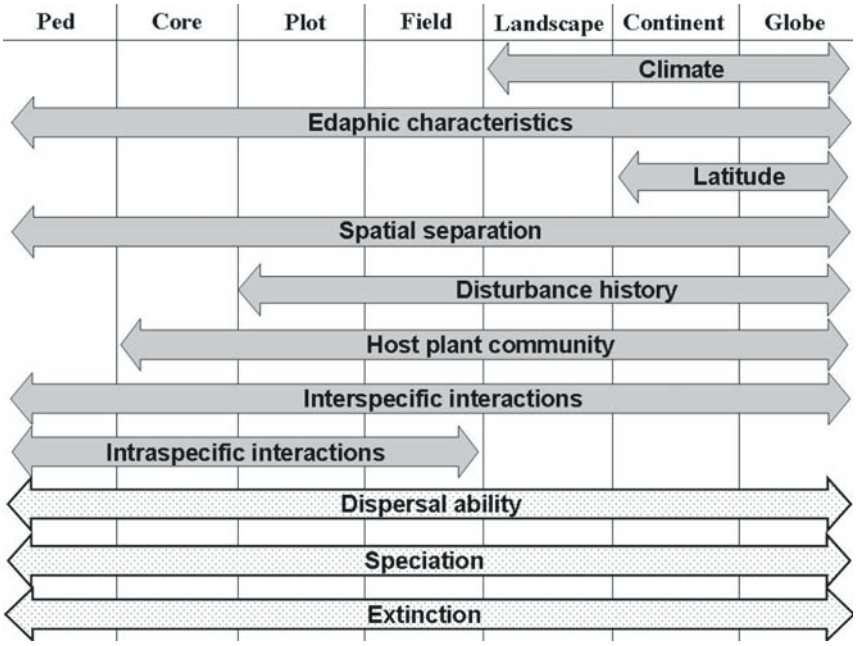

Fig. 6 The spatial scales where factors that create variation in species distributions are relevant. *Shaded arrows* indicate external abiotic and biotic forces and *dotted arrows* represent intrinsic properties of AM fungal species

many peds. It has been argued that, although scientists have been studying soil organisms for several decades, very little is known about soil structural and biological dynamics at the core and ped scales (Crawford et al. 2005). In contrast, the plot and field scales are comparatively well studied in soil ecology. Larger than the field scale are the landscape, continent, and finally global scales. Each of the major factors identified to influence geographic distributions are relevant along a gradient of these spatial scales from ped to globe.

Climatic factors, such as temperature and precipitation, likely only influence AM fungal distributions at landscape, continent, and global scales. Edaphic characteristics, such as nutrient dynamics and texture, could influence AM fungal geographic distributions on all spatial scales. For example, edaphic properties could change from the outer edges of a ped to the inner portions of a ped, influencing spatial distributions of fungi at the ped scale. Latitude likely only influences AM fungal distributions at the landscape, continent and global scales because solar radiation generally does not differ at scales smaller than the landscape scale. On the other hand, spatial separation could contribute to variation in fungal distributions at all scales; just as oceans create separation between multiple continents at the global scale, space between two soil aggregates creates separation between peds. Disturbance

history likely influences the geographic distributions of the Glomeromycota only from the plot scale to the global scale because natural and anthropogenic disturbances generally do not differ at scales smaller than the plot-level. Similarly, host plant community could affect AM fungal distributions at all spatial scales except the ped scale because plant influences on AM fungi likely do not differ within a single soil aggregate. Interspecific interactions, such as those among AM fungi of different species or between AM fungi and other organisms, could influence the distributions of Glomeromycota at all spatial scales because organisms of all sizes – from large ungulates to soil bacteria and archaea – could influence geographic distributions of AM fungal species. However, intraspecific interactions likely only influence distributions at smaller scales from the ped to the plot. By definition, interactions between individuals of the same fungal species occur in close proximity. The inherent dispersal ability of an AM fungal species is likely to be an important factor in determining patterns of distribution at all spatial scales. Different species could vary in their ability to disperse across a ped as well as across multiple continents. And, finally, although the mechanisms of speciation and extinction for Glomeromycotan fungi are poorly understood, these processes have the potential to affect geographic distributions of species at all spatial scales. The biogeographical challenge lies in identifying how species distributions vary by scale, which mechanisms are most important at various scales, and which scales are relevant for the management and conservation of Glomeromycotan fungi.

2.8 Modeling Approaches

Conservation biologists recognize the need to prioritize protection and restoration efforts, which has led to technological advances in methods for mapping and quantifying the distributions and diversity of plants and animals. Furthermore, advances in statistical modeling and mathematical theory have led to the ability to make ecological inferences at spatial scales where experimental manipulations are impractical or impossible. Advances in computing power and the development of specialized software for the capture and manipulation of geographic information systems (GIS) now make digital spatial data easily accessible and publicly available. Unfortunately, few of these technological advances have been employed for the use of determining the spatial distributions or biodiversity of microbes. Studies in microbial biogeography would particularly benefit from these modeling techniques because geographic distributions of microorganisms, especially at the smallest and largest scales, cannot be easily observed. Advanced techniques in spatial scaling represent an important frontier in microbial biogeography, becoming an essential tool in the microbial biogeographer's toolbox.

Traditional methods used to determine species distributions connect localities where a given species is present and assume that the species is uniformly distributed in that area. Researchers establish a grid, determine the presence or absence of a species in each cell of the grid (i.e., occupancy) and create a distribution map by

connecting the occupied cells. The degree to which communities located close to each other are also similar to each other, or spatial autocorrelation, can be estimated using semivariogram analysis. Semivariograms represent the average variance between two samples taken at increasing distances from one another (Schlesinger et al. 1996). However, certain methods can overestimate the interior area occupied by a species and underestimate areas inhabited outside known points (Sanchez-Cordero et al. 2004). Furthermore, biologists would agree that the probability of detection of most species is not equal to 1 and therefore methods that estimate detection probability are preferred. Modern occupancy modeling is GIS-based and incorporates information regarding detection probability to estimate abundance and occupancy of a species in the geographic range of interest (MacKenzie et al. 2006).

Another approach to estimate species distributions is ecological niche modeling which correlates occupancy data with environmental factors to predict the potential distribution of a species (Sanchez-Cordero et al. 2004). Distribution maps can be constructed using GIS-layers of environmental conditions and resources that represent relevant niche characteristics. The produced distribution maps represent the fundamental niche of a species, or the geographic region that the species could potentially occupy (Grinnell 1917; Hutchinson 1959). Distribution maps of individual species can then be summed to produce maps of biodiversity hotspots, and predicted distributions are validated with field data. Examples of methods for ecological niche modeling are GAP analysis (Scott et al. 2002), BIOCLIM (Nix 1986), and DOMAIN (Carpenter et al. 1993), which all incorporate occupancy data and environmental data such as habitat types, climatic conditions, and biophysical attributes to create potential distribution maps. Other methods that have high predictive success, such as genetic algorithm for rule-set prediction (GARP) and boosted regression trees, use evolutionary computing systems (Elith et al. 2006; Guisan et al. 2007). These techniques determine the environmental factors that best describe an ecological niche for a species through an iterative process of evaluating random subsets of data (Stockwell and Noble 1992; Stockwell and Peters 1999). Recently, GARP has had success in predicting the locations of biodiversity hotspots for plants and animals, the extent of invasive species, as well as the geography of disease transmission (Raxworthy et al. 2003; Peterson et al. 2003; Peterson 2006). To our knowledge, ecological niche modeling has never been applied to modeling geographic distributions of Glomeromycotan fungi.

Beyond mapping AM fungal species distributions, it is important to understand the dominant mechanisms that drive biogeographical patterns of species. Are observed patterns controlled more by contemporary environmental conditions or past historical events? Older methods in statistical modeling can be applied in new ways to address biogeographical questions. For example, dissimilarity matrices constructed from various types of data matrices can be compared using a Mantel test to determine whether AM fungal communities that are similar with respect to structure and composition also have similar geographic locations (i.e., autocorrelation) or environmental conditions (Mantel 1967; Martiny et al. 2006). Furthermore, analyses using partial Mantel tests can be used to parse out the amount of variation in the community that is explained by geographic distance versus other environmental

variables (Martiny et al. 2006; Smouse et al. 1986). In a similar approach, structural equation modeling could be used to simultaneously analyze several different mechanisms that generate biogeographical patterns. In structural equation modeling, a priori conceptual models presenting causal relationships between systems of interrelated variables (such as those presented in Figs. 1–5) are tested using field data. By comparing the covariance structure of data that is predicted by the model with the actual covariance structure of the data, we can test the fit of the model and fail to disprove it (Grace and Pugesek 1998; McCune and Grace 2002; Shipley 2000). At spatial scales where manipulative experiments are impossible, these methods can be used in observational studies to improve the level of inference that can be made about the dominant mechanisms behind biogeographical patterns of species.

Null models of ecological phenomena are another approach for studying ecological patterns using observational data. Null modeling involves building a mathematical model of a pattern that would result given the absence of a process of interest. An example, and potential AM fungal biogeographic application, of null modeling is co-occurrence analysis. In co-occurrence analysis, observational data of the composition of multiple communities in the form of presence absence matrices is compared to random assemblages that are generated from the original dataset using a null model algorithm. This null model algorithm is based on the principal that species with overlapping niches cannot co-exist indefinitely (Diamond 1975; Connor and Simberloff 1979; Weiher and Keddy 2001). Software combining the null model algorithm with a randomization procedure generates a standardized index of co-occurrence (Gotelli and Entsminger 2004), which provides a quantitative means of assessing the degree to which the community is structured by competition.

3 Conclusions

A better understanding of Glomeromycotan biogeography is essential for the conservation of AM fungal species and the services they provide in nearly all ecosystems worldwide. Many factors, from external abiotic and biotic forces to intrinsic properties of species, have the potential to create variation in the geographic distributions of AM fungal species. Interactions among these factors generate the many complex mechanisms that control the geographic distributions of AM fungi. Now is an exciting time to study Glomeromycotan biogeography because new approaches using molecular genetics, modeling, and mathematics are helping to reveal the many possible mechanisms that create non-random spatial patterns. As the global human population approaches 7 billion, impacts on natural ecosystems and habitat losses are increasing. Identifying hotspots of AM fungal biodiversity, regions or environmental conditions that sustain diverse communities of AM fungi, will aid in preserving the many important functions that they provide in ecosystems.

References

Abbott LK, Robson AD (1985) The effect of soil pH on the formation of vesicular arbuscular mycorrhizas by two species of *Glomus*. Aust J Soil Res 23:253–261

Abbott LK, Robson AD (1991) Factors influencing the occurrence of vesicular-arbuscular mycorrhizas. Agric Ecosyst Environ 35:121–150

Abbott LK, Robson AD, Hall IR (1983) Introduction of vesicular arbuscular mycorrhizal fungi into agricultural soils. Aust J Agric Res 34:741–749

Allen AP, Gillooly JF, Savage VM, Brown JH (2006) Kinetic effects of temperature on rates of genetic divergence and speciation. Proc Natl Acad Sci USA 103:9130–9135

Allen EB, Chambers JC, Connor KF, Allen MF, Brown RW (1987) Natural reestablishment of mycorrhizae in disturbed alpine ecosystems. Arct Alp Res 19:11–20

Allen EB, Allen MF, Helm D, Trappe J, Molina R, Rincon E (1995) Patterns and regulation of mycorrhizal plant and fungal diversity. In: Collins H, Robertson G, Klung M (eds) The significance and regulation of soil biodiversity. Kluwer, Dordrecht, pp 47–62

Allen EB, Rincon E, Allen MF, Perez-Jimenez A, Huante P (1998) Disturbance and seasonal dynamics of mycorrhizae in a tropical deciduous forest in Mexico. Biotropica 30:261–274

Allen MF (1991) The ecology of mycorrhizae. Cambridge University Press, New York

Andersson L (1990) The driving force: species concepts and ecology. Taxon 39:375–382

Avise JC (2000) Phylogeography: the history and formation of species. Harvard University Press, Cambridge, Mass.

Baas Becking LGM (1930) Salt effects on swarmers of *Dunaliella viridis*. Teod J Gen Physiol 14:765–763

Baas-Becking LGM (1934) Geobiologie of inleiding tot de milieukunde. Van Stockkum & Zoon, The Hague

Bala A, Murphy P, Giller KE (2003) Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the tropics. Mol Ecol 12:917–930

Batten KM, Scow KM, Davies KF, Harrison SP (2006) Two invasive plants alter soil microbial community composition in serpentine grasslands. Biol Invasions 8:217–230

Belnap J, Phillips SL (2001) Soil biota in an ungrazed grassland: response to annual grass (*Bromus tectorum*) invasion. Ecol Appl 11:1261–1275

Bentivenga SP, Hetrick BAD (1991) Relationship between mycorrhizal activity, burning, and plant productivity in a tallgrass prairie. Can J Bot 69:2597–2602

Bentivenga SP, Bever JD, Morton JB (1997) Genetic variation of morphological characters within a single isolate of the endomycorrhizal fungus *Glomus clarum* (Glomaceae). Am J Bot 84:1211–1216

Bever JD, Morton JB (1999) Heritable variation and mechanisms of inheritance of spore shape within a population of *Scutellospora pellucida*, an arbuscular mycorrhizal fungus. Am J Bot 86:1209–1216

Bever JD, Wang M (2005) Arbuscular mycorrhizal fungi: Hyphal fusion and multigenomic structure. Nature 433:E3-E4

Bever J, Westover K, Antonovics J (1997) Incorporating the soil community into plant population dynamics: the utility of the feedback approach. J Ecol 85:1–13

Bever JD, Schultz PA, Pringle A, Morton JB (2001) Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. Bioscience 51:923–931

Brown A, Bledsoe C (1996) Spatial and temporal dynamics of mycorrhizas in *Jaumea carnosa*, a tidal saltmarsh holophyte. J Ecol 84:703–715

Brown, JH (1995) Macroecology. The University of Chicago Press, Chicago, Ill.

Brown JH, Gibson AC (1983) Biogeography. Mosby, St. Louis, Mo.

Brown JH, Mehlman DW, Stevens GC (1995) Spatial variation in abundance. Ecology 76:2028–2043

Bunn RA, Zabinski CA (2003) Arbuscular mycorrhizae in thermal-influenced soils in Yellowstone National Park. West North Am Nat 63:409–415

Carpenter G, Gillison AN, Winter J (1993) DOMAIN: a flexible modelling procedure for mapping potential distributions of plants and animals. Biodivers Cons 2:667–680

Chapela IH, Osher LJ, Horton TR, Henn MR (2001) Ectomycorrhizal fungi introduced with exotic pine plantations induce soil carbon depletion. Soil Biol Biochem 33:1733–1740
Chaudhary VB (2006). Functions of arbuscular mycorrhizal fungi at community and ecosystem scales in semi-arid environments. M.S. Thesis, Northern Arizona University
Cho JC, Tiedje JM (2000) Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Appl Environ Microbiol 66:5448–5456
Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265
Clapp JP, Helgason T, Daniell TJ, Young JPW (2002) Genetic studies of the structure and diversity of arbuscular mycorrhizal fungal communities. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 201–224
Connell JH, Slayter RO (1977) Mechanisms of succession in natural communities and their role in community stability and organization. Am Nat 111:1119–1144
Connor EF, Simberloff D (1979). The assembly of species communities: chance or competition? Ecology 60:1132–1140
Crawford JW, Harris JA, Ritz K, Young IM (2005) Towards an evolutionary ecology of life in soil. Trends Ecol Evol 20:81–87
Daily GC, Matson PA, Vitousek PM (1997) Ecosystem services supplied by soil. In: Daily GC (ed) Nature's services: societal dependence on natural ecosystems, Island Press, Washington, D.C., pp 113–132
de Candolle AP (1820) Essai elementaire de geographie botanique. Levrault, Paris
de la Providencia IE, de Souza FA, Fernandez F, Delmas NS, Declerck S (2005) Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. New Phytol 165:261–71
de Wit R, Bouvier T (2006) "Everything is everywhere, but, the environment selects"; what did Baas-Becking and Beijerinck really say? Environ Microbiol 8:755–748
Dhillion SS, Anderson RC (1993) Seasonal dynamics of dominant species of arbuscular mycorrhizae in burned and unburned sand prairies. Can J Bot 71:1625–1630
Diamond JM (1975) Assembly of species communities. In: Cody ML, Diamond JM (eds) Ecology and evolution of communities. Harvard University Press, Cambridge, Mass., pp 342–444
Egerton-Warburton LM, Allen EB (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecol Appl 10:484–496
Egerton-Warburton LM, Johnson NC, Allen EB (2007) Mycorrhizal community dynamics following nitrogen fertilization: a cross-site test in five grasslands. Ecol Monogr 77(4):527–544
Elith J, Graham CH, Anderson RP, Dudík M, Ferrier S, Guisan A et al. (2006) Novel methods improve prediction of species' distributions from occurrence data. Ecography 29:129–151
Eom AH, Hartnett DC, Wilson GWT, Figge DA (1999) Effects of fire, mowing and fertilizer amendments on arbuscular mycorrhizas in tallgrass prairie. Am Midl Nat 142:55–69
Eom AH, Wilson GWT, Hartnett DC (2001) Effects of ungulate grazers on arbuscular mycorrhizal symbiosis and fungal community structure in tallgrass prairie. Mycologia. 93:233–242
Ettema CH, Wardle DA (2002) Spatial soil ecology. Trends Ecol Evol 17:177–183
Falush D, Wirth T, Linz B, Pritchard JK, Stephens M, Kidd M, Blaser MJ, Graham DY, Vacher S, Perez-Perez GI, Yamaoka Y, Megraud F, Otto K, Reichard U, Katzowitsch E, Wang X, Achtman M, Suerbaum S (2003) Traces of human migrations in Helicobacter pylori populations. Science 299:1582–1585
Feldmann F (1998) The strain-inherent variability of arbuscular mycorrhizal effectiveness. Symbiosis 25:131–143
Fitter AH (2005) Darkness visible: reflections on underground ecology. J Ecol 93:231–243
FitzJohn RG, Dickie IA (2007) TRAMPR: AN R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. Mol Ecol Notes 7: 583–587
Franklin RB, Mills AL (2003) Multi-scale variation in spatial heterogeneity for microbial community structure in an eastern Virginia agricultural field. FEMS Microbiol Ecol 44:335–346

Fry JC (1990) Direct methods and biomass estimation. In: Grigorova R, Norris JR (eds) Methods in microbiology, vol 22. Academic, London, pp 41–85
Gange AC, VK Brown (2002) Actions and interactions of soil invertebrates and arbuscular mycorrhizal fungi in affecting the structure of plant communities. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 321–344
Gehring CA, Whitham TG (2002) Mycorrhizae-herbivore interactions: population and community consequences. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 297–320
Gehring CA, Wolf JE, Theimer TC (2002) Terrestrial vertebrates promote arbuscular mycorrhizal fungal diversity and inoculum potential in a rain forest soil. Ecol Lett 5:540–548
Gemma JN, Koske RE, Carreiro M (1989) Seasonal dynamics of selected species of V-A mycorrhizal fungi in a sand dune. Mycol Res 92:317–321
Gianinazzi-Pearson V, Diem HG (1982) Endomycorrhizae in the Tropics. In: Dommergues YR, Diem HG (eds) Microbiology of tropical soils - implications in soil management. Nijhoff, The Hague, pp 209–251
Giovannetti M (1985) Seasonal variations of vesicular-arbuscular mycorrhizas and endogonaceous spores in a maritime sand dune. Trans Br Mycol Soc 84:679–684
Gotelli NJ, Entsminger GL (2004) EcoSim: null models software for ecology. Version 7. Acquired Intelligence and Kesey-Bear, Jericho, Vt.
Grace JB (2006) Structural equation modeling and natural systems. Cambridge University Press, New York
Grace JB, Pugesek BH (1998) On the use of path analysis and related procedures for the investigation of ecological problems. Am Nat 152:151–159
Green JL, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. Trends Ecol Evol 21:501–507
Green JL, Holmes AJ, Westoby M, Oliver I, Briscoe D, Dangerfield M, Gillings M, Beattie A (2004) Spatial scaling of microbial eukaryote diversity. Nature 432:747–750
Grinnell, J (1917) The niche relationships of the California Thrasher. Auk 34:427–433
Guisan A, Zimmermann NE, Elith J, Graham CH, Phillips S, Peterson AT (2007) What matters for predicting spatial distributions of trees: techniques, data, or species' characteristics? Ecol Monogr (in press)
Hart MM, Klironomos JN (2002) Diversity of arbuscular mycorrhizal fungi and ecosystem functioning. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 225–242
Hart MM, Reader RJ (2002) Taxonomic basis for variation in the colonization strategy of arbuscular mycorrhizal fungi. New Phytol 153:335–344
Hawkes CV, Wren IF, Herman DJ, Firestone MK (2005) Plant invasion alters nitrogen cycling by modifying the soil nitrifying community. Ecol Lett 8:976–985
Helgason T, Fitter A (2005) The ecology and evolution of the arbuscular mycorrhizal fungi. Mycologist 19:96–101
Herrera-Peraza RA, Cuenca G, Walker C (2001) *Scutellospora crenulata*, a new species of Glomales from La Gran Sabana, Venezuela. Can J Bot 79:674–678
Hillebrand H (2004) On the generality of the latitudinal diversity gradient. Am Nat 163:192–211.
Horner-Devine MC, Lage M, Hughes JB, Bohannan BJM (2004) A taxa-area relationship for bacteria. Nature 432:750–753
Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871
Hutchinson GE (1959) Homage to Santa Rosalia; or, why are there so many kinds of animals? Am Nat 93:145–159
Janos DP, Sahley CT, Emmons LH (1995) Rodent dispersal of vesicular-arbuscular mycorrhizal fungi in Amazonian Peru. Ecology 76:1852–1858
Jasper DA, Abbott LK, Robson AD (1991) The effect of soil disturbance on vesicular-arbuscular mycorrhizal fungi in soils from different vegetation types. New Phytol 118:471–476

Johnson NC, Pfleger FL (1992) Vesicular-arbuscular mycorrhizae and cultural stresses. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in sustainable agriculture. American Society of Agronomy, Madison, Wis., pp 71–99

Johnson NC, Tilman D, Wedin D (1992) Plant and soil controls on mycorrhizal fungal communities. Ecology 73:2034–2042

Johnson D, Vanerkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ (2004) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytol 161:503–515

Johnson NC, Wedin DA (1997) Soil carbon, nutrients and mycorrhizal fungal communities during conversion of a dry tropical forest to grassland. Ecol Appl 7:171–182

Johnson NC, Rowland DL, Corkidi L, Egerton-Warburton LM, Allen EB (2003) Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84:1895–1908

Johnson NC, Hoeksema JD, Bever JD, Chaudhary VB, Gehring C, Klironomos J, Koide R, Miller RM, Moore J, Moutoglis P, Schwartz M, Simard S, Swenson W, Umbanhowar J, Wilson G, Zabinski C (2006) From Lilliput to Brobdingnag: extending models of mycorrhizal function across scales. Bioscience 56:889–900

Klironomos JN (2003) Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84:2292–2301

Koske RE (1987) Distribution of vesicular-arbuscular mycorrhizal fungi along a latitudinal temperature gradient. Mycologia 79:55–68

Kramadibrata K, Walker C, Schwarzott D, Schüssler A (2000) A new species of *Scutellospora* with a coiled germination shield. Ann Bot 86:21–27

Lambert DH, Cole H, Baker DE (1980) Adaptation of vesicular-arbuscular mycorrhizae to edaphic factors. New Phytol 85:513–540

Lekberg Y, Koide RT, Rohr JR, Aldrich-Wolfe L, Morton JB (2007) Role of niche restrictions and dispersal in the composition of arbuscular mycorrhizal fungal communities. J Ecol 95:95–105

Lenski RE, Travisano M (1994) Dynamics of adaptation diversification: a 10,000-generation experiment with bacterial populations. Proc Natl Acad Sci USA 91:6808–6814

Liu WT, Marsh TL, Cheng H, Forney LJ (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl Environ Microbiol 63:4516–4522

Lomolino MV (2004) Conservation biogeography. In: Lomolino MV, Heaney LR (eds) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass., pp 293–296

Lomolino MV, Heaney LR (eds) (2004) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass.

Lovelock CE, Andersen K, Morton JB (2003) Arbuscular mycorrhizal communities in tropical forests are affected by host tree species and environment. Oecologia 135:268–279

MacKenzie DI (2006) Occupancy estimation and modeling: inferring patterns and dynamics of species. Elsevier, Amsterdam

Magurran AE (1988) Ecological diversity and its measurement. Princeton, N.J.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Res 27:209–220

Martiny JBH, Bohannan B, Brown J, Colwell R, Fuhrman J, Green J, Horner-Devine MC, Kane M, Krumins J, Kuske C, Morin P, Naeem S, Ovreas L, Reysenbach AL, Smith V, Staley L (2006) Microbial biogeography: putting microorganisms on the map. Nature Rev Microbiol 4:102–112

McCune B, Grace JB (2002) Analysis of Ecological Communities. MjM Software Design, Gleneden Beach, Or.

McIlveen WD, Cole H (1976) Spore dispersal of Endogonaceae by worms, ants, wasps and birds. Can J Bot 54:1486–1489

Miller RM (1987) Mycorrhizae and succession.. In: Jordan WR, Gilpin ME, Aber JD (eds) Restoration ecology: a synthetic approach to ecological research. Cambridge University Press, Cambridge, pp 205–220

Miller RM, Jastrow JD (2000) Mycorrhizal fungi influence soil structure. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 3–18

Miller SP, Bever JD (1999) Distribution of arbuscular mycorrhizal fungi in stands of the wetland grass *Panicum hemitomon* along a wide hydrologic gradient. Oecologia 119:586–592

Morrall RAA (1974) Soil microfungi associated with aspen in Saskatchewan: synecology and quantitative analysis. Can J Bot 52:1803–1817

Morton JB, Bentivenga SP (1994). Levels of diversity in endomycorrhizal fungi (Glomales, Zygomycetes) and their role in defining taxonomic and non-taxonomic groups. Plant Soil 159:47–59

Morton JB, Bentivenga SP, Bever JD (1995) Discovery, measurement, and interpretation of diversity in arbuscular endomycorrhizal fungi (Glomales, Zygomycetes). Can J Bot 73:s25-s32

Mummey DL, Rillig MC (2006) The invasive plant species Centaurea maculosa alters arbuscular mycorrhizal fungal communities in the field. Plant Soil 288:81–90

Nekola JC, White PS (1999) The distance decay of similarity in biogeography and ecology. J Biogeogr 26:867–878

Newsham K, Fitter A, Watkinson A (1995) Multi-functionality and biodiversity in arbuscular mycorrhizas. Trends Ecol Evol 10:407–411

Öpik M, Moora M, Liira J, Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J Ecol 94:778–790

Pawlowska TE (2005) Genetic processes in arbuscular mycorrhizal fungi. FEMS Microbiol Lett 251:185–192

Pawlowska TE, Taylor JW (2005) Arbuscular mycorrhizal fungi: hyphal fusion and multigenomic structure (reply). Nature 433:E4

Peterson AT (2006) Ecologic niche modeling and spatial patterns of disease transmission. Emerging Infectious Diseases 12:1822–1826

Peterson AT, Papes M, Kluza DA (2003) Predicting the potential invasive distributions of four alien plant species in North America. Weed Science 51:863–868

Pimm SL, Russell GJ, Gittleman JL, Brooks TM (1995) The Future of Biodiversity. Science 269:347

Pirozynski KA, Malloch DW (1975) The origin of land plants: a matter of mycotrophism. Biosystems 6:153–164

Ponder F (1980) Rabbits and grasshoppers: vectors of encomycorrhizal fungi on new coal mine spoil. North Central Forest Experiment Station, USDA Forest Service Research Note No. NE-250. Washington, DC

Porter WM, Robson AD, Abbott LK (1987) Factors controlling the distribution of vesicular-arbuscular mycorrhizal fungi in relation to soil pH. J Appl Ecol 24:663–672

Pringle A, Bever JD (2002) Divergent phenologies may facilitate the coexistence of arbuscular mycorrhizal fungi in a North Carolina grassland. Am J Bot 89:1439–1446

Pringle A, Taylor JW (2002) The fitness of filamentous fungi. Trends Microbiol 10:474–481

Pringle A, Vellinga EC (2006) Last chance to know? Using literature to explore the biogeography and invasion biology of the death cap mushroom *Amanita phalloides* (Vaill. ex Fr.: Fr.). Biol Invasions 8:1131–1144

Rabatin SC, Rhodes LH (1982) *Acaulospora bireticulata* inside oribatid mites. Mycologia 74:859–861

Rainey PB, Travisano M (1998) Adaptive radiation in a heterogeneous environment. Nature 394:69–72

Raxworthy CJ, Martínez-Meyer E, Horning N, Nussbaum RA, Schneider GE, Ortega-Huerta MA, Peterson AT (2003) Predicting distributions of known and unknown reptile species in Madagascar. Nature 426:837–841

Read DJ (1991) Mycorrhizas in ecosystems. Experientia 47:376–391

Read DJ, Koucheki HK, Hodgson T (1976) Vesicular-arbuscular mycorrhizae in natural vegetation ecosystems. New Phytol 77:641–653
Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921
Riddle BR, Funk V (2004) Phylogeography and diversification. In: Lomolino MV, Heaney LR (eds) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass., pp 87–92
Riddle BR, DJ Hafner (2004) The past and future roles of phylogeography in historical biogeography. In: Lomolino MV, Heaney LR (eds) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass., pp 93–110
Rillig MC (2004a) Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecol Lett 7:740–754
Rillig MC (2004b) Arbuscular mycorrhizae, glomalin and soil quality. Can J Soil Sci 84:355–363
Rogers YH, Venter JC (2005) Genomics: massively parallel sequencing. Nature 437:376–380
Rohde K (1992) Latitudinal gradients in species diversity: the search for the primary cause. Oikos 65:514–527
Rosendahl S, Stukenbrock EH (2004) Community structure of arbuscular mycorrhizal fungi in undisturbed vegetation revealed by analysis of LSU rDNA sequences. Mol Ecol 13:3179–3186
Rosenzweig ML (1992) Species diversity gradients: we know more and less than we thought. J Mammal 73:715–730
Rothwell FM, Holt C (1978) Vesicular-arbuscular mycorrhizae established with *Glomus fasciculatus* spores isolated from feces of cricetine mice. Northeast Forest Experiment Station, USDA Forest Service Research Note No. NE-259. Washington, DC
Sanchez-Cordero V, Munguia M, Peterson AT (2004) GIS-based predictive biogeography in the context of conservation. In: Lomolino MV, Heaney LR (eds) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass., pp 311–324
Sanders I (2002) Ecology and evolution of multigenomic arbuscular mycorrhizal fungi. Am Nat 160:S128–S141
Sanders I, Fitter A (1992) Evidence for differential responses between host-fungus combinations of vesicular-arbuscular mycorrhizas from a grassland. Mycol Res 96:415–419
Schenck NC, Perez Y (1990) Manual for the identification of VA mycorrhizal fungi, 3rd edn. Synergistic Publications, Gainesville, Fla.
Schlesinger WH, Raikes JA, Hartley AE, Cross AF (1996) On the spatial pattern of soil nutrients in desert ecosystems. Ecology 77:364–374
Schultz PA, Miller RM, Jastrow JD, Rivetta CV, Bever JD (2001). Evidence of a mycorrhizal mechanism for the adaptation of *Andropogon gerardii* (Poaceae) to high- and low-nutrient prairies. Am J Bot 88:1650–1656
Schüßler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1321
Schwalbach MS, Fuhrman JA (2005) Wide-ranging abundances of aerobic, anoxygenic phototrophic bacteria in the world ocean revealed by epifluorescence and quantitative PCR. Limnol Oceanogr 50:620–628
Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal inoculum. Ecol Lett 9:501–515
Scotese, CR (2002) Cenozoic and mesozoic paleogeography: changing terrestrial biogeographic pathways. In: Lomolino MV, Heaney LR (eds) Frontiers of biogeography: new directions in the geography of nature. Sinauer, Sunderland, Mass., pp 9–26
Scott JM, Heglund PJ, Morrison ML (eds) (2002) Predicting species occurrences: issues of accuracy and scale. Island Press, Washington, D.C
Shipley B (2000) Cause and correlation in biology: a user's guide to path analysis, structural equations and causal inference. Cambridge University Press, Cambridge, UK

Sieverding E (1990) Ecology of VAM fungi in tropical agrosystems. Agric Ecosyst Environ 29:369–390
Siguenza C, Corkidi L, Allen EB (2006a) Feedbacks of soil inoculum of mycorrhial fungi altered by N deposition on the growth of a native shrub and an invasive annual grass. Plant Soil 286:153–165
Siguenza C, Crowley DE, Allen EB (2006B) Soil microorganisms of a native shrub and exotic grasses along a N deposition gradient. Appl Soil Ecol 32:13–26
Simon L, Bousquet J, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:67–69
Simpson GG (1951) The species concept. Evolution 5:285–298
Singer MJ, Munns DN (2002) Soils: an introduction. Pearson, N.J.
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, New York
Smouse PE, Long JC, Sokal RR (1986) Multiple regression and correlation extensions of the Mantel test of matrix correspondence. Syst Zool 35:627–632
Staddon WJ, Trevors JT, Duchesne LC, Colombo CA (1998) Soil microbial diversity and community structure across a climatic gradient in western Canada. Biodivers Cons 7:1081–1092
Stockwell DRB, Noble IR (1992) Induction of sets of rules from animal distribution data: a robust and informative method of analysis. Math Computers Simulation 33:385–390.
Stockwell DRB, Peters DP (1999) The GARP modelling system: problems and solutions to automated spatial prediction. Int J Geogr Inf Syst 13:143–158
Stutz JC, Morton JB (1996) Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. Can J Bot 74:1883–1889
Tews J, Brose U, Grimm V, Tielbörger K, Wichmann MC, Schwager M, Jeltsch F (2004) Animal species diversity driven by habitat heterogenity/diversity: the importance of keystone structures. J Biogeogr 31:79–92
Tiedje JM (1995) Approaches to the comprehensive evaluation of prokaryote diversity of a habitat. In: Allsopp D, Colwell RR, Hawksworth DL (eds) Microbial diversity and ecosystem function. CAB I, Wallingford, UK, pp 73–88
Torsvik V, Goksoyr J, Daae FL (1990) High diversity in DNA of soil bacteria. Appl Environ Microbiol 56:782–787
Trappe JM (1987) Phylogenetic and ecological aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In: Safir GR (ed) Ecophysiology of VA mycorrhizal plants. CRC Press, Boca Raton, Fla., pp 5–25
van der Heijden M, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engle R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72
Walker C (1992) Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales) - a possible way forward. Agronomie 12:887–897
Warner NJ, Allen MF, MacMahon JA (1987) Dispersal agents of vesicular-arbuscular mycorrhizal fungi in a disturbed arid ecosystem. Mycologia 79:721–730
Weiher E, Keddy P (2001) Ecological assembly rules: perspective, advances and retreats. Cambridge University Press, Cambridge, U.K.
Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E (1998) Quantifying threats to imperiled species in the United States: assessing the relative importance of habitat destruction, alien species, pollution, overexploitation, and disease. BioScience 48:607–615
Wilson EO (1988) Biodiversity. National Academy Press, Washington, D.C.
Wolfe B, Mummey D, Rillig M, Klironomos J (2007) Small-scale spatial heterogeneity of arbuscular mycorrhizal fungal abundance and community composition in a wetland plant community. Mycorrhiza 17:175–183
Wright SD, Keeling J, Gillman LN (2006) The road from Santa Rosalia: a faster tempo of evolution in tropical climates. Proc Natl Acad Sci USA 103:7718–7722
Zhang N, Blackwell M (2002) Population structure of dogwood anthracnose fungus. Phytopathology 92:1276–1283

Arbuscular Mycorrhiza of Endangered Plant Species: Potential Impacts on Restoration Strategies

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1 Introduction

A site-specific flora and fauna characterize natural habitats. However, the plant diversity in ecosystems rapidly decreases more than often due to anthropogenic activities. The destruction of habitats leads to a decrease in ecosystem diversity, plant diversity and, in worst cases, to the extinction of plant species (Tilman et al. 1994). About 3% of the described plant species of the world are considered to be threatened, although according to some estimation this value may reach a maximum of 70%. In any case, over the years the number of plant species belonging to different categories of threat (critical, endangered, and vulnerable) is rising continually (IUCN 2006). In nature symbiotic associations between plant roots and mycorrhizal fungi appear to be the norm (Smith and Read 2008). Numerous experiments have shown the positive influence of arbuscular mycorrhizal fungi (AMF) on plant fitness by enhancing the uptake of essential nutrients, the resistance against root pathogens, and the tolerance of environmental stress like drought and heavy metals. The stability and diversity of plant communities have been shown to be influenced by the microbial community in soil including mycorrhizal fungi, and vice versa plant communities affect arbuscular mycorrhizal fungi. These interactions underline the importance of a functioning soil micro-flora including mycorrhizal fungi for a well functioning ecosystem and therefore indicate the potential significance of fungal symbionts of plants for restoration practices (Haselwandter 1997), especially as there seems to be a defined relationship between single types of AMF and their plant hosts (van der Heijden 1998a).

2 Diversity of AMF

Natural habitats not only harbor a diverse plant cover, but also a diverse soil community with a variety of different microorganisms including AMF. From the number of only about 200 morphologically identified species a very low diversity of possible interactions

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between plant species and AMF could be inferred. Nevertheless, the morphological as well as the inter-and intraspecific genetic and functional diversity of AMF in different ecosystems suggests a much wider variety of AMF in certain habitats. Brundrett (1991) found a very diverse composition of fungal spore populations present in different habitats in a tropical region of Australia. However, differences in fungal species diversity can even be discovered on a more local scale. Blaszkowski (1994) found a difference in the frequency of arbuscular mycorrhizal genera in the root zones of various plant species collected from a dune habitat in Poland. The differences can be associated with plant families as well as the plant species itself. On the basis of morphological criteria in a long-term study Bever et al. (2001) identified 37 AMF species, about a third of which have not been described previously. On the basis of molecular data, AMF colonizing the roots of the endangered African pencil cedar, *Juniperus procera*, were shown to differ from any sequence types found in other ecosystems (Wubet et al. 2006). In a tropical wetland, Husband et al. (2002) demonstrated with molecular tools that the AMF diversity of the plant species *Faramea occidentalis* and *Tetragastris panamensis* differed between sites and in time. A study of Pringle and Bever (2002) revealed that AMF are very diverse on a very fine scale, both in space and in time, and that the sporulation rate depends on the host plant. Sýkorová et al. (2007) showed in an alpine grassland that the diversity of the AMF community in roots depends on the host species rather than on the site. In addition, Santos-González et al. (2007) found different AMF communities due to the host. A survey on the distribution of AM fungi found in ecosystems around the world has demonstrated that about 50% of the taxa have been reported from one single site and that about one-third appear only on one host plant species, while on the other hand there are also species which seem to be globally distributed (Öpik et al. 2006).

It is clear that there is a close relationship between the plant cover and the diversity of fungal species present in soil. The soil community often lacks attention although it can significantly influence the performance of plant species (Kardol et al. 2006). Different experiments confirm the influence of AM fungal diversity upon plant community structure and vice versa. Grime et al. (1987) showed that the presence of AM fungi in soil, together with other parameters, is increasing the diversity of the plant cover. More specifically, Gange et al. (1993) revealed that the application of fungicides, and as a result the apparent lower inoculation potential, negatively influenced plant species composition. The AMF colonization intensity of the test plant *Veronica persica* was lower at the sites where fungicides were applied than on nontreated sites. The number of plant species per site was lower in plots treated with fungicides than in nontreated soil. In contrast, O'Connor et al. (2002) reported no change in the species richness of the plant cover, but an increase in evenness of a site treated with benomyl. Vice versa, the composition of the plant cover as well as soil factors are affecting the AM fungal community in soil (Johnson et al. 1992, 2003). These interactions are due to the different benefits plants and fungi derive from each other. Plants appear to benefit differently by various AM fungi, but also AMF react differently to a plant host regarding, e.g., sporulation (Pringle and Bever 2002). The work of van der Heijden et al. (1998b) points out the relationship between certain fungal isolates and plant species. Plant species of a calcareous grassland were selected and inoculated with single or a combination of different AM fungal strains. The plant species benefited differently from the fungal strains regarding biomass production. Vogelsang et al. (2006)

demonstrated that the plant diversity and plant productivity is rather more dependent on the identity of the fungus than on the actual diversity of AMF in soil, which makes it clear that a loss of diversity, i.e., certain strains, can lead to a change in the plant community structure. However, it needs to be stressed that there appears to be a high intraspecific functional diversity within AMF species (Munkvold et al. 2004; Koch et al. 2004). As plant species and the soil microflora including the mycorrhizal fungi are linked together, a disturbance of either community is bound to negatively influence the other and hence ecosystem biodiversity and structure. However, the relationship between host plant and fungi shows a continuum of potential interactions ranging from mutualism to parasitism (Jones and Smith 2004). Francis and Read (1995) examined the effect of mycorrhization on selected plants growing in a microcosm. *Plantago lancelota* grew best when inoculated with mycorrhizal fungi whereas other plant species like *Rumex acetosella, Spergula avensis* and *Reseda luteola* performed best in the nonmycorrhizal compartment. Mycorrhizal relationships between plant species and fungi are dependent on the mycorrhizal dependency of a certain plant species and the environmental conditions of rhizosphere and plant and soil communities (Johnson et al. 1997; Bever and Schultz 2005).

3 Factors Causing a Change in the AMF Community

Human impacts, like change of agricultural management practices, erosion, top soil disturbance, mining, tillage, as well as the change of chemical and physical soil properties, lead to a reduction of the diversity of the AMF community in soil (Abbott and Robson 1991), especially as different genera are reacting differently to such disturbances (Dodd 2000) and, thus, can vanish from the fungal community. Helgason et al. (1998) compared the diversity of AM fungal species present in woodland and an agricultural soil. This study revealed that the AMF species diversity at the agricultural site is much lower than in woodland soil. The fungus *Glomus mosseae* was the dominant species in the agricultural soil, whereas in woodland soil species of the genera *Glomus, Acaulospora* and *Scutellospora* were found in a more diverse mixture. This agrees with the results of Jansa et al. (2002) who tested the effect of tillage on the AMF community in soil which supported mostly *Glomus* spp. Oehl et al. (2005) compared different agricultural practices and concluded that the agriculturally intensively used maize field harbored a less diverse AMF community in the top soil layer than extensive grasslands. Heavy tillage seems to lead to a reduction of the diversity of AMF in agricultural sites in comparison to less intensively treated fields. In degraded forest soils, Jha et al. (1992) found a significantly smaller AMF population than in less disturbed forest habitats. They concluded that disturbance of the soil (and vegetation) negatively affects the microbial population including AMF spores. Miller et al. (1985) investigated the effects of stockpiling during mining on soil organisms and observed that the potential for mycorrhizal colonization was reduced over time of stockpiling, which was partly due to the reduction of mycorrhizal plant species present in the soil during stockpiling. Another major factor disturbing AMF in soil derives from impact of toxic elements. In a contaminated soil harboring remnants of chemical industrial waste,

Vallino et al. (2006) found a very low diversity of AMF in roots of *Solidago gigantea*. The dominating fungi identified in plant roots were members of the genus *Glomus*. However, the diversity of AMF present in soil has not been examined. Turnau et al. (2001) analyzed the fungal community of a polluted site in Poland and discovered only the genus *Glomus* as being present in soil in the form of spores and in plant roots as identified by molecular tools. This corresponds with data from Whitfield et al. (2004) who found a predominance of the genus *Glomus* in roots of *Thymus polytrichus* grown in heavy metal polluted soil. Disturbance of soil communities as well as changes in chemical and physical soil parameters can be considered as main reasons for changes in the composition and the diversity of AMF in soil.

4 Identification and Assessment of AMF Colonization

In order to be able to produce inoculum suitable for a given plant species it is important to know its mycorrhizal status. Classical microscopical methods have to be applied in order to gain an overview whether the plant species is mycorrhizal or not. The sampling site soil has to be analyzed in order to allow for a morphological classification of the fungi present in the rhizosphere of the plant and to assess the diversity of the AMF present in the soil environment. However, classical methods are inappropriate for analysis of the correlation between fungal symbionts in the roots and those present in the soil. In the last couple of years, various molecular methods have been developed in order to identify the fungi colonizing a plant root or to molecularly identify the fungi present in soil. These methods include SSCP (Kjøller and Rosendahl 2000), RAPD (Lanfranco et al. 1995), AFLP (Rosendahl and Taylor 1997) and the specific amplification of different regions (SSU, LSU or ITS) of the ribosomal DNA. So, a holistic approach in analyzing both the roots and the soil would be the best in order to gain the knowledge necessary for development of suitable inoculum if needed. One example using this approach was the study of the plant species *Hyacinthoides non-scripta*. Merryweather and Fitter (1998a, 1998b) and Clapp et al. (1995) have morphologically assessed the mycorrhizal status of this plant species and evaluated the diversity of fungal symbionts present in the roots of the plant species by molecular biological methods. A comparable study focused on two species of *Pulsatilla*, one of which is endangered. The mycorrhizal status was investigated with molecular tools, and experiments on seed establishment, and inoculation effects were carried out to enlighten the ecology of these species (Öpik et al. 2003; Moora et al. 2004).

Progress in mycorrhiza research, especially in the field of applied ecology, is hampered by the nature of the AMF, which still holds many open questions and which makes AMF difficult to work with (Rosendahl 2008, Young 2008). At present both morphological as well as molecular analyses are not sufficient to elucidate precisely the exact diversity of AMF in soil. Currently, about 200 arbuscular mycorrhizal species are described and their number will continue to rise in future years. However, techniques to sample and cultivate arbuscular mycorrhizal fungi are still inadequate to reflect the

potential diversity in a given habitat. Bever et al. (2001) pointed out that, even with different methods including repeated sampling throughout the vegetation period as well as the establishment of trap cultures, not all AMF species could be depicted in soil. Some species are either not present as spores or not capable of sporulating under certain soil conditions. Furthermore, some species possibly cannot be trapped in cultures due to a potential selectivity of bait plant species used as well as the soil conditions and temperature regime employed. Clapp et al. (1995) could not detect AMF spores of all the fungal partners of understory plants as identified by molecular methods. This shows the inadequacy of the classical morphological methods. However, more or less the same is true for molecular biological methods, which have been established in order to identify the fungi colonizing plant roots. Identification of AMF is mostly based on the amplification of parts of the rDNA either with unspecific (Clapp et al. 1995; Helgason et al. 1998) or specific primers for families, genera or species of AMF (see, e.g., Redecker 2000; Renker et al. 2003; Kjøller and Rosendahl 2000, 2001; Millner et al. 2001). The general primers often do not amplify all the members of the Glomeromycota, and therefore do not reflect the true diversity of all the fungi involved in colonizing a plant root. Problems in clearly defining AMF diversity may derive from the lack of a clear species concept and the polymorphism of the marker genes (Redecker et al. 2003). Wubet et al. (2006) stated that with the molecular methods currently available it is difficult to perfectly assess the diversity of natural ecosystems. A major problem is the genetic variability of AMF within a single species. Studies on the diversity of AMF propose high genetic diversity between and within single species (Clapp et al. 2001; Koch et al. 2004). Therefore, AMF sequences obtained from one root sample are often difficult to relate to one single species of AMF (Sanders 2004a), resulting in an undefined diversity of AMF in plant roots. Until the ecology of AMF itself has been enlightened more thoroughly, molecular studies on the diversity of AMF in natural ecosystems are subject to such limitations. However, these flaws in the classical and molecular methodology should not keep us from working in natural ecosystems and trying to elucidate the relationship between plant and fungus, in particular through further improvement of the methods employed.

5 Inoculum: Indigenous Versus Exotic Fungi

Regulatory steps like establishment of seed banks and revegetation projects still do not cover protection and conservation of AMF associated with a given plant species in need of protection. A prerequisite for successful reestablishment of plant species is a functioning soil microflora including a mycorrhizal network (Hart and Klironomos 2002) especially as AMF help to sustain good conditions for plant health and soil fertility (Haselwandter 1997; Turnau and Haselwandter 2002). Mycorrhizal mycelial networks often constitute 20–30% of total soil microbial biomass and influence soil, plant, and ecosystem processes (Leake et al. 2004). Requena et al. (2001) reported on the importance of microbial propagules for the formation of a dynamic rhizosphere and the successful establishment of the plant cover. In this experiment, *Anthyllis cytisoides* was inoculated either with an exotic AMF species or a mixture of indigenous AMF species. In the long run, the

indigenous AMF species were much more efficient in promoting plant growth and improving soil parameters than *Glomus intraradices* deriving from a different habitat. As a consequence, a soil environment as natural as possible for plants should be established in order to provide support for growth in the new environment. Even endangered and common plant species from the genus *Pulsatilla* inoculated with native AMF from different habitats show specificity with regard to the inoculum they prefer (Moora et al. 2004). In a growth experiment with a local mycotrophic plant species, native isolates enhanced growth more than nonnative isolates, especially in interaction with local site conditions including the microflora (Oliviera et al. 2005). As inadequate inoculum should be avoided due to possible negative effects on plant species and the plant community, it is necessary to analyze the fungal community associated with an endangered plant species in its natural environment as long as such a threatened plant is still available in an undisturbed condition. This is the only chance and basis for designing suitable inoculum when needed.

The use of appropriate inoculum was discussed in a recent review by Schwartz et al. (2006), who suggested international guidelines on inoculum management in order to decrease the possible negative effects by using inadequate inoculum. The application of inappropriate inoculum could result in the upcoming or survival of invasive plant species, the outcompetition of native fungal strains and the introduction of pests in case the inoculum contains pathogens.

6 Restoration

Reestablishment of endangered plant species and restoration of destroyed landscapes or specific plant communities need careful consideration concerning the inoculum and the plant species used, especially as there are interactions between plant species and AMF which decide whether a plant species receives positive or negative support by a certain AMF strain under certain environmental conditions (van der Heijden 1998b; Johnson et al. 1997; Francis and Read 1995). An experiment carried out by Herrera et al. (1993) showed that reclamation of a desertified area was most successful with native plant species, which could be improved by inoculation with selected soil microbes including AMF. The same is true for *Pulsatilla* spp. inoculated with two different kinds of natural inoculum associated with the different species. The endangered as well as the common species of *Pulsatilla* performed best with the inoculum found in their natural environment (Moora et al. 2004). These examples show that it is very important to know the AMF associates of a given plant species in order to be able to supply the best inoculum for best plant performance. Klironomos (2003) conducted an inoculation experiment with native and exotic AMF species. He concluded that for establishing a diverse plant community it is important to use a mix of a locally adapted AMF community, as plant species benefited most from certain AMF species. Therefore, it may be advisable to use not only one single AMF species for restoration practices, but to include a bunch of AMF species in order to be able to support a diverse plant cover (Vogelsang et al. 2006).

In a long-term experiment, Johnson (1998) discovered that *Salsola kali*, an early successional plant, and *Panicum virgatum*, a late successional plant, showed the highest yield at unamended, uninoculated plots and amended, AMF-inoculated plots, respectively. Plant communities change over time with regard to plant cover, nutrient level and mycorrhizal associations. Restoration strategies should also take into account the successional stage of the plant community which needs to be restored, as certain early succession plant communities are deprived of AMF and may harbor a plant community not dependent on AMF, but maybe other fungal colonizers like dark septate endophytes.

Destruction of a habitat often leads to a decrease in the inoculum potential present at a given site. The change or the destruction of the plant cover can lead to a decrease in sporulation, lower amount of colonized roots and a destruction of the hyphal network. When the endangered plant species *Arnica montana*, growing in turf-cut sites, was supplied with suitable AMF inoculum mycorrhizal colonization, seedling growth and establishment were significantly enhanced (Vergeer et al. 2006).

Unfortunately, the AMF are still not known well enough to be able to draw a detailed picture of their biology. There is still the question to be raised how the fungi are organized and whether the genomic information is organized in genetically different (Sanders et al. 1995; Hijri et al. 1999; Sanders 1999; Kuhn et al. 2001; Bever and Wang 2005) or identical nuclei (Pawlowska and Taylor 2004; Pawlowska 2005). Furthermore, it still has to be revealed whether AMF are defined by clonality (Stukenbrock and Rosendahl 2005) or by recombination (Vandenkoornhuyse 2001). Moreover, it would be interesting to know which genes are expressed under certain environmental conditions. Which and how many ecotypes can result from one fungal strain? These questions define at least some of the problems concerning inoculation of plant species with AMF. Additional hurdles might arise from the limits inherent to the production of inoculum (Sanders 2004b). In this context, it is important to know the preferred colonization strategies of AMF species (i.e., by means of spores, colonized root fragments or extraradical hyphae), as it was shown that different fungal genera prefer to colonize plant species from different sources of inoculum (Klironomos and Hart 2002). Another problem encountered is that not all of the AMF species are amenable to mass production (Schwartz et al. 2006).

7 Interactions with Other Soil Microorganisms

Another type of colonization, which is found in nearly any ecosystem, is that by dark septate endophytes (DSE). The fungi seem either to be dominant in different habitats (Haselwandter and Read 1980; Read and Haselwandter 1981; Barrow and Aaltonen 2001), in specific successional stages of plant communities (Cázares et al. 2005), or colonize together with mycorrhizal fungi including, e.g., AMF (Ruotsalainen et al. 2002; Fuchs and Haselwandter 2004). To date, no absolute host specificity has been shown, but preferences for certain plant species, plant communities or habitats do exist (Sieber and Grünig 2006). Similar to AMF, there

is a continuum of interactions between DSE and plants covering a wide range from parasitism to mutualism (Jumpponen and Trappe 1998). Thus, it is not surprising that the question was raised already whether or not DSE can be considered as mycorrhizal fungi (Jumpponen 2001). DSE fungi belong to a wide range of different ascomycetes. This is reflected by the diverse spectrum of the biology of DSE (Girlanda et al. 2006) and may help to explain why the knowledge of their ecology is still scanty. It is clear, that typical DSE like *Phialocephala fortinii* Wang & Wilcox can promote plant growth. In growth experiments, two *Carex* species were inoculated with the DSE strains C1 and C2 (Haselwandter and Read 1982), at least one of which (i.e. C2 = CC3 in Jumpponen and Trappe 1998; Bartholdy et al. 2001) was identified as *Phialocephala fortinii* s.l. (Grünig, personal communication). Both strains tested have been shown to enhance the phosphorus content of two and the biomass of one *Carex* species. Similar growth experiments were carried out with the grass *Vulpia ciliata* ssp. *ambigua* inoculated with *Phialophora graminicola*, also positively influencing plant performance (Newsham 1999). In addition to phosphorus uptake by plants, DSE may also be involved in stimulation of the uptake of other nutrients. Bartholdy et al. (2001) have demonstrated for a range of *Phialocephala fortinii* strains the production of hydroxamate siderophores. Such compounds chelating ferric iron are known for their potential to enhance the iron uptake by plants (Haselwandter 1995; Haselwandter and Winkelmann 2007).

Thus, it is clear that at least some strains of DSE may stimulate plant growth and improve-plant nutrition, and therefore can be considered as mutualists. Hence, it is important to also consider these fungi for restoration projects, especially in environments where they may act as key colonizers of plants. In destructed habitats, DSE could act as fungal root associates for certain plant species prior to the establishment of other mycorrhizal communities (Sieber and Grünig 2006). However, the true relationship between a given plant species and DSE would need to be experimentally analyzed case-by-case in order to select for fungal strains acting beneficial upon plant growth and fitness.

8 Mycorrhization of Endangered Medicinal Plants: A Special Case

Threatened plant species which have the potential of being used for medical purposes deserve special interest in particular because, at least in some areas, such a use and an increasing demand and market for the respective products may enhance the risk that they become extinct. It is known that secondary metabolite contents in root and shoot tissues of AM plants may increase over nonmycorrhizal plants (Strack et al. 2003). Both, *Glomus macrocarpum* and *G. fasciculatum* significantly enhanced the concentration of the terpenoid artemisinin in *Artemisia annua* leaves (Kapoor et al. 2007). Mycorrhizal plants of *Castanospermum australe* contained higher amounts of the alkaloid castanospermine in their leaves in comparison to nonmycorrhizal controls with a differential effect of AM fungal inocula present (Abu-Zeyad et al. 1999). In the case of *Coleus forskohlii* inoculation with most of the AM fungal species tested led to higher contents of the alkaloid forskolin in the

roots compared to uninoculated plants (Sailo and Bagyaraj 2005). In India, *C. forskohlii* is an important medicinal plant, which has become an endangered species due to the continuous collection of roots.

In general, the mycorrhizal status of a great variety of medicinal plants has not so far been thoroughly investigated. However, there are some studies reporting on the colonization of medicinal plants by arbuscular mycorrhizal or ectomycorrhizal fungi and dark septate root endophytes. Lakshmipathy et al. (2003) assessed the AM colonization intensity of five threatened medicinal plant species growing in southern India, and found varying colonization intensities depending on the plant species and site investigated, and related to soil pH, P content and phosphatase activity. The AMF spore density present in the rhizosphere soil of three endangered medicinal plant species sampled in the Indian Thar Desert appeared to be positively correlated with soil pH and organic carbon and negatively with Olson's phosphorus content (Panwar and Tarafdar 2006). Muthukumar et al. (2007) examined more than 100 different medicinal and aromatic plant species in southern India and found about 74% of the plant species examined to be colonized by AMF, ca. 36% by dark septate endophytes, and approximately 28% by both types of fungal root associates. In the same study, the arbuscular mycorrhizal morphology appeared to be related to the plant growth form, with a predominance of *Arum*-type mycorrhiza in herbaceous and annual plants, while the *Paris*-type was more prominent in trees. Frequency of root associations with dark septate endophytes was higher in herbaceous and perennial plants in comparison to other growth forms of medicinal and aromatic plants. Many species of the hemiparasitic plant genus *Pedicularis* are known to possess pharmacological effects and therefore are used as components of, e.g., Chinese traditional herbal drugs. Li and Guan (2007) have demonstrated that arbuscular and ectomycorrhizal fungi as well as dark septate endophytes, with the latter appearing to be the most common root colonizers achieving highest colonization levels, colonize *Pedicularis* species.

Undoubtedly, the studies selected as examples above and similar investigations provide valuable information on the mycorrhizal status of endangered medicinal plants. However, their value is limited because they do not provide insight with regard to the fungal species, eco- or genotype of a species which is actually colonizing the root system of a given plant species. In that respect, the only one or at least one of very few exceptions is the study by Wubet et al. (2003) in which molecular methods were applied for determination of the diversity of arbuscular mycorrhizal fungi in the threatened medicinal tree species *Prunus africana* growing in dry Afromontane forests of Ethiopia. This study provided evidence for a high AM fungal diversity with a predominance of *Glomus* types.

9 Outlook

Despite inherent limitations, investigations as described above are of paramount importance with regard to the development of suitable inoculum for the establishment of endangered plants for a number of reasons. It is known, for example, that (1) the AMF biodiversity as displayed by the presence of spores in rhizosphere

soil does not necessarily reflect the AMF biodiversity present in plant roots, and (2) different AMF, which functionally can be very diverse, can simultaneously colonize the very same root segment. Once characterized, specific fungal root symbionts of threatened plants can be isolated and selected as well as tested for their efficacy. Thus, such an approach will provide the best basis for the production of inoculum to be used for the reestablishment of endangered species or cultivation of selected medicinal plants. Indigenous inoculum should be favored over exotic fungi, as the consequences of introduction of nonnative fungi on plant species and plant community structure cannot be precisely predicted. Hence, a sound strategy for the conservation of threatened plant species ought to comprise both plant seeds as well as fungal root associates like appropriate AMF strains, in analogy to an approach suggested for long-term preservation of rare orchids (Batty et al. 2001).

A holistic view on ecosystem functioning is bound to include DSE and their potential impact on mycorrhiza formation and plant succession. It may be important to incorporate these fungi in reestablishment practices as they might improve plant fitness, and might either be the prime and dominant fungal root associates or can concurrently colonize a plant root together with AMF. Beneficial interactions in the rhizosphere include those between AMF and rhizobia or plant growth-promoting rhizobacteria (PGPR) as well as between PGPR and rhizobia (Saxena et al. 2006). It would also be interesting to know the effect of DSE on arbuscular mycorrhiza formation and of AMF on colonization by DSE.

The mycorhizosphere comprises a community of different organisms including plants, mycorrhizal fungi and other soil microorganisms, and soil fauna (Timonen and Marschner 2006). This habitat harbors bacteria which are able to support plants by either directly promoting plant growth or indirectly by facilitating AMF colonization of plant species (Barea et al. 2002; Artursson et al. 2006). Requena et al. (1997) have evaluated the interactions between AMF, *Rhizobium* and PGPR in a re-vegetation experiment with *Anthyllis cytisoides*. Results of this study showed that different kinds of inoculation mixtures differently promoted plant performance. Thus a holistic approach with regard to the design of microbial inocula to be used in reestablishment programmes for endangered plant species appears to be justified.

Human impact leading to ecosystem destruction necessitates the development of restoration strategies. Looking at the great number of various parameters determining a plant community, the inclusion of soil microorganisms in restoration practices is indispensable. Future AMF research is desperately needed for enlightening the AMF biology, optimizing the AMF inoculum production and elucidating the precise relationship between endangered plant species and their soil environment for successful reestablishment of plant species in a new environment or in a destroyed ecosystem under restoration.

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References

Abbott LK, Robson AD (1991) Factors influencing the occurrence of vesicular-arbuscular mycorrhizas. Agric Ecosyst Environ 35:121–150

Abu-Zeyad R, Khan AG, Khoo C (1999) Occurrence of arbuscular mycorrhiza in *Castanospermum australe* A. Cunn. & C. Fraser and effects on growth and production of castanospermine. Mycorrhiza 9:111–117

Artursson V, Finlay RD, Jansson JK (2006) Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. Environ Microbiol 8:1–10

Barea JM, Azcón R, Azcón-Aguilar C (2002) Mycorrhizosphere interactions to improve plant fitness and soil quality. Antonie van Leeuwenhoek 81:343–351

Barrow JR, Aaltonen RE (2001) Evaluation of the internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity. Mycorrhiza 11:199–205

Bartholdy BA, Berreck M, Haselwandter K (2001) Hydroxamate siderophore synthesis by *Phialocephala fortinii*, a typical dark septate fungal root endophyte. BioMetals 14:33–42

Batty AL, Dixon KW, Brundrett M, Sivasithamparam K (2001) Long-term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. Aust J Bot 49:619–628

Bever JD, Schultz PA (2005) Mechanisms of arbuscular mycorrhizal mediation of plant-plant interaction. In: Dighton J, White JF, Oudemans P: The fungal community: its organization and role in the ecosystem. Taylor & Francis, Boca Raton, Fla., pp 443–459

Bever JD, Wang M (2005) Hyphal fusion and multigenomic structure. Nature 433:E3-E4

Bever JD, Schultz PA, Pringle A, Morton JB (2001) Arbuscular mycorrhizal fungi: more diverse than meets the eye, and the ecological tale of why. BioScience 51:923–931

Blaszkowski J (1994) Arbuscular fungi and mycorrhizae (Glomales) of the Hel Peninsula, Poland. Mycorrhiza 5:71–88

Brundrett M (1991) Mycorrhizas in natural ecosystems. Adv Ecol Res 21:171–313

Cázares E, Trappe JM, Jumpponen A (2005) Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. Mycorrhiza 15:405–416

Clapp JP, Young JPW, Merryweather JW, Fitter AH (1995) Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. New Phytol 130:259–265

Clapp JP, Rodriguez A, Dodd JC (2001) Inter- and intra-isolate rRNA large subunit variation in *Glomus coronatum* spores. New Phytol 149:539–554

Dodd JC (2000) The role of arbuscular mycorrhizal fungi in agro- and natural ecosystems. Outlook Agric 29:63–70

Francis R, Read DJ (1995) Mutualism and antagonism in the mycorrhizal symbiosis, with special reference to impacts on plant community structure. Can J Bot 73:S1301-S1309

Fuchs B, Haselwandter K (2004) Red list plants: colonization by arbuscular mycorrhizal fungi and dark septate endophytes. Mycorrhiza 14:277–281

Gange AC, Brown VK, Sinclair GS (1993) Vesicular-arbuscular mycorrhizal fungi: a determinant of plant community structure in early succession. Funct Ecol 7:616–622

Girlanda M, Perotto S, Luppi AM (2006) Molecular diversity and ecological roles of mycorrhiza-associated sterile fungal endophytes in Mediterranean ecosystems. In: Schulz B, Boyle C, Sieber T (eds) Microbial root endophytes. Soil Biology Series. Springer, Heidelberg, pp 207–226

Grime JP, Mackey JML, Hillier SH, Read DJ (1987) Floristic diversity in a model system using experimental microcosms. Nature 328:420–422

Hart MM, Klironomos JN (2002) Diversity of arbuscular mycorrhizal fungi and ecosystem functioning. In: van der Heijden MGA, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 225–242

Haselwandter K (1995) Mycorrhizal fungi: siderophore production. Crit Rev Biotechnol 15:287–291

Haselwandter K (1997) Soil micro-organisms, mycorrhiza, and restoration ecology. In: Urbanska KM, Webb NR, Edwards PJ (eds) Restoration ecology and sustainable development. Cambridge University Press: Cambridge, pp 65–80

Haselwandter K, Read DJ (1980) Fungal Associations of roots of dominant and sub-dominant plants in high-alpine vegetation systems with special reference to mycorrhiza. Oecologia 45:57–62

Haselwandter K, Read DJ (1982) The significance of a roots-fungus association in two *Carex* species of high-alpine plant communities. Oecologia 53:352–354

Haselwandter K, Winkelmann G (2007) Siderophores of symbiotic fungi. In: S.B. Chincholkar SB, Varma A (eds) Microbial Siderphores. Soil Biology Series Springer: Heidelberg, pp 91–103

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431

Herrera MA, Salamanca CP, Barea JM (1993) Inoculation of woody legumes with selected arbuscular mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. Appl Environ Microbiol 59:129–133

Hijri M, Hosny M, van Tuinen D, Dulieu H (1999) Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fungal Genet Biol 26:141–151

Husband R, Herre EA, Turner SL, Gallery R, Young JPW (2002) Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Mol Ecol 11:2669–2678

IUCN (2006) 2006 IUCN Red List of threatened species. International Union for Conservation of Nature and Natural Resources. http://www.iucnredlist.org/info/stats [29.12.2006]

Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jha DK, Sharma GD, Mishra RR (1992) Ecology of soil microflora and mycorrhizal symbionts in degraded forests at two altitudes. Biol Fertil Soils 12:272–278

Johnson D, Vandenkoornhuyse PJ, Leake JR, Gilbert L, Booth RE, Grime JP, Young JPW, Read DJ (2003) Plant communities affect arbuscular mycorrhizal fungal diversity and community composition in grassland microcosms. New Phytol 161:503–515

Johnson NC (1998) Responses of *Salsola kali* and *Panicum virgatum* to mycorrhizal fungi, phosphorus and soil organic matter: implications for reclamation. J Appl Ecol 35:86–94

Johnson NC, Tilman D, Wedin D (1992) Plant and soil controls on mycorrhizal fungal communities. Ecology 73:2034–2042

Johnson NC, Graham JH, Smith FA (1997) Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol 135:575–585

Jones MD, Smith SE (2004) Exploring functional definitions of mycorrhizas: are mycorrhizas always mutualisms? Can J Bot 82:1089–1109

Jumpponen A (2001) Dark septate endopyhtes – are they mycorrhizal? Mycorrhiza 11:207–211

Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol 140:295–310

Kardol P, Bezemer TM, van der Putten WH (2006) Temporal variation in plant-soil feedback controls succession. Ecol Lett 9:1080–1088

Kapoor R, Chaudhary V, Bhatnagar AK (2007) Effects of arbuscular mycorrhiza and phosphorus application on artemisinin concentration in *Artemisia annua* L. Mycorrhiza 17:581–587

Kjøller R, Rosendahl S (2000) Detection of arbuscular mycorrhizal fungi (Glomales) in roots by nested PCR and SSCP (single stranded conformation polymorphism). Plant Soil 226:189–196

Kjøller R, Rosendahl S (2001) Molecular diversity of glomalean (arbuscular mycorrhizal) fungi determined as distinct *Glomus* specific DNA sequences from roots of field grown peas. Mycol Res 105:1027–1032

Klironomos JN (2003) Variation in plant response to native and exotic arbuscular mycorrhizal fungi. Ecology 84:2292–2301

Klironomos JN, Hart MM (2002) Colonization of roots by arbuscular mycorrhizal fungi using different sources of inoculum. Mycorrhiza 12:181–184

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet J, Sanders IR (2004) High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. Proc Natl Acad Sci USA 101:2369–2374

Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. Nature 414:745–748

Lakshmipathy A, Gowda B, Bagyaraj DJ (2003) VA mycorrhizal colonization pattern in RET medicinal plants (*Mammea suriga, Saraca asoca, Garcinia* spp., *Embelia ribes* and *Calamus* sp.) in different parts of Karnataka. Asian J Microbiol Biotechnol Environ Sci 5:505–508

Lanfranco L, Wyss P, Marzachi C, Bonfante P (1995) Generation of RAPD-PCR primers for the identification of isolates of *Glomus mosseae*, an arbuscular mycorrhizal fungus. Mol Ecol 4:61–68

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Can J Bot 82:1016–1045

Li AR, Guan KY (2007) Mycorrhizal and dark septate endophytic fungi of *Pedicularis* species from northwest of Yunnan Province, China. Mycorrhiza 17:103–109

Merryweather J, Fitter A (1998a) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* I. Diversity of fungal taxa. New Phytol 138:117–129

Merryweather J, Fitter A (1998b) The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* II. Seasonal and spatial patterns of fungal populations. New Phytol 138:131–142

Miller RM, Carnes BA, Moorman TB (1985) Factors influencing survival of vesicular-arbuscular mycorrhiza propagules during topsoil storage. J Appl Ecol 22:259–266

Millner PD, Mulbry WW, Reynolds SL (2001) Taxon-specific oligonucleotide primers for detection of two ancient endomycorrhizal fungi, *Glomus occultum* and *Glomus brasilianum*. FEMS Microbiol Lett 196:165–170

Moora M, Öpik M, Sen R, Zobel M (2004) Native arbuscular mycorrhizal fungal communities differentially influence the seedling performance of rare and common *Pulsatilla* species. Funct Ecol 18:554–562

Munkvold L, Kjøller R, Vestberg M, Rosendahl S, Jakobsen I (2004) High functional diversity within species of arbuscular mycorrhizal fungi. New Phytol 164:357–364

Muthukumar T, Senthilkumar M, Rajangam M, Udaiyan K (2007) Arbuscular mycorrhizal morphology and dark septate fungal associations in medicinal and aromatic plants of Western Ghats, Southern India. Mycorrhiza 17:11–24

Newsham (1999) *Phialophora graminicola*, a dark septate fungus, is a beneficial associate of the grass *Vulpia ciliata* ssp. *ambigua*. New Phytol 144:517–524

O'Connor PJ, Smith SE, Smith FA (2002) Arbuscular mycorrhizas influence plant diversity and community structure in a semiarid herbland. New Phytol 154:209–218

Oehl F, Sieverding E, Ineichen K, Ris E-A, Boller T, Wiemken A (2005) Community structure of arbuscular mycorrhizal fungi at different soil depths in extensively and intensively managed agroecosystems. New Phytol 165:273–283

Oliveira RS, Vosátka M, Dodd JC, Castro PML (2005) Studies on the diversity of arbuscular mycorrhizal fungi and the efficacy of two native isolates in a highly alkaline anthropogenic sediment. Mycorrhiza 16:23–31

Öpik M, Moora M, Liira J, Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J Ecol 94:778–790

Öpik M, Moora M, Liira J, Kõljalg U, Zobel M, Sen R (2003) Divergent arbuscular mycorrhizal fungal communities colonize roots of *Pulsatilla* spp. in boreal Scots pine forest and grassland soils. New Phytol 160:581–593

Panwar J, Tarafdar JC (2006) Distribution of three endangered medicinal plant species and their colonization with arbuscular mycorrhizal fungi. J Arid Environ 65:337–350

Pawlowska TE (2005) Genetic processes in arbuscular mycorrhizal fungi. FEMS Microbiol Lett 251:185–192

Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. Nature 427:733–737

Pringle A, Bever JD (2002) Divergent phenologies may facilitate the coexistence of arbuscular mycorrhizal fungi in a North Carolina grassland. Am J Bot 89:1439–1446

Read DJ, Haselwandter K (1981) Observations on the mycorrhizal status of some alpine plant communities. New Phytol 88:341–352

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Redecker D, Hijri I, Wiemken A (2003) Molecular identification of arbuscular mycorrhizal fungi in roots: perspectives and problems. Folia Geobot 38:113–124

Renker C, Heinrichs J, Kaldorf M, Buscot F (2003) Combining nested PCR and restriction digest of the internal transcribed spacer region to characterize arbuscular mycorrhizal fungi on roots from the field. Mycorrhiza 13:191–198

Requena N, Jimenez I, Toro M, Barea JM (1997) Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* spp. in the rhizosphere of *Anthyllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. New Phytol 136:667–677

Requena N, Perez-Solis E, Azcón-Aguilar C, Jeffries P, Barea JM (2001) Management of indigenous plant-microbe symbioses aids restoration of desertified ecosystems. Appl Environ Microbiol 67:495–498

Rosendahl S (2008) Communities, populations and individuals of arbuscular mycorrhizal fungi. New Phytol 178:253–266

Rosendahl S, Taylor JW (1997) Development of multiple genetic markers for studies of genetic variation in arbuscular mycorrhizal fungi using AFLP™. Mol Ecol 6:821–829

Ruotsalainen AL, Väre H, Vestberg M (2002) Seasonality of root fungal colonization in low-alpine herbs. Mycorrhiza 12:29–36

Sailo GL, Bagyaraj DJ (2005) Influence of different AM-fungi on the growth, nutrition and forskolin content of *Coleus forskohlii*. Mycol Res 109:795–798.

Sanders IR (1999) No sex please, we're fungi. Nature 399:737–739

Sanders IR (2004a) Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? New Phyotol 164:415–418

Sanders IR (2004b) Intraspecific genetic variation in arbuscular mycorrhizal fungi and its consequences for molecular biology, ecology, and development of inoculum. Can J Bot 82:1057–1062

Sanders IR, Alt M, Groppe K, Boller T, Wiemken A (1995) Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytol 130:419–427

Santos-González JC, Finlay RD, Tehler A (2007) Seasonal dynamics of arbuscular mycorrhizal fungal communities in roots in a seminatural grassland. Appl Environ Microbiol 73:5613–5623

Saxena AK, Shende R, Grover M (2006) Interactions among beneficial microorganisms. In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere. Soil Biology Series. Springer, Heidelberg, pp 121–137

Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515

Sieber TN, Grünig CR (2006) Biodiversity of fungal root-endophyte communities and populations, in particular of the dark septate endophyte *Phialocephala fortinii* s.l. In: Schulz B, Boyle C, Sieber T (eds) Microbial root endophytes. Soil Biology Series. Springer, Heidelberg, pp 107–132

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd ed. Academic: New York

Strack D, Fester T, Hause B, Schliemann W, Walter MH (2003) Arbuscular mycorrhiza: biological, chemical and molecular aspects. J Chem Ecol 29:1955–1979

Stukenbrock EH, Rosendahl S (2005) Clonal diversity and population genetic structure of arbuscular mycorrhizal fungi (*Glomus* spp.) studied by multilocus genotyping of single spores. Mol Ecol 14:743–752

Sýkorová Z, Wiemken A, Redecker D (2007) Cooccurring *Gentiana verna* and *Gentiana acaulis* and their neighboring plants in two Swiss upper montane meadows harbor distinct arbuscular mycorrhizal fungal communities. Appl Environ Microbiol 73:5426–5434

Tilman D, May RM, Lehman CL, Nowak MA (1994) Habitat destruction and the extinction debt. Nature 371:65–66

Timonen S, Marschner P (2006) Mycorhizosphere concept. In: Mukerji KG, Manoharachary C, Singh J (eds) Microbial activity in the rhizosphere. Soil Biology Series. Springer, Heidelberg, pp 155–172

Turnau K, Haselwandter K (2002) Arbuscular mycorrhizal fungi, an essential component of soil microflora in ecosystem restoration. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhäuser, Basel, pp 137–149

Turnau K, Ryszka P, Gianinazzi-Pearson V, van Tuinen D (2001) Identification of arbuscular mycorrhizal fungi in soils and roots of plants colonizing zinc wastes in southern Poland. Mycorrhiza 10:169–174

Vallino M, Massa N, Lumini E, Bianciotto V, Berta G, Bonfante P (2006) Assessment of arbuscular mycorrhizal fungal diversity in roots of *Solidago gigantea* growing in a polluted soil in Northern Italy. Environ Microbiol 8:971–983

Vandenkoornhuyse P, Leyval C, Bonnin I (2001) High genetic diversity in arbuscular mycorrhizal fungi: evidence for recombination events. Heredity 87:243–253

Van der Heijden MGA, Boller T, Wiemken A, Sanders IR (1998a) Different arbuscular mycorrhizal fungal species are potential determinants of plant community structure. Ecology 79:2082–2091

Van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998b) Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396:69–72

Vergeer P, van den Berg LJL, Baar J, Ouborg NJ, Roelofs JGM (2006) The effect of turf cutting on plant and arbuscular mycorrhizal spore recolonisation: implications for heathland restoration. Biol Conserv 129:226–235

Vogelsang KM, Reynolds HL, Bever JD (2006) Mycorrhizal fungal identity and richness determine the diversity and productivity of a tallgrass prairie system. New Phytol 172:554–462

Whitfield L, Richards AJ, Rimmer DL (2004) Relationships between soil heavy metal concentration and mycorrhizal colonisation in *Thymus polytrichus* in northern England. Mycorrhiza 14:55–62

Wubet T, Weiß M, Kottke I, Teketay D, Oberwinkler F (2003) Molecular diversity of arbuscular mycorrhizal fungi in *Prunus africana*, an endangered medicinal tree species in dry Afromontane forests of Ethiopia. New Phytol 161:517–528

Wubet T, Weiß M, Kottke I, Teketay D, Oberwinkler F (2006) Phylogenetic analysis of nuclear small subunit rDNA sequences suggests that the endangered African Pencil Cedar, *Juniperus procera*, is associated with distinct members of *Glomeraceae*. Mycol Res 110:1059–1069

Young JPW (2008) The genetic diversity of intraterrestrial aliens. New Phytol 178:465–468

Community Developmental Patterns and Ecological Functions of Ectomycorrhizal Fungi: Implications from Primary Succession

Kazuhide Nara

1 Introduction

Ectomycorrhizal fungal (EMF) communities are extremely species-rich in most forest ecosystems (Horton and Bruns 2001). The number of EMF species can often reach more than 100 at even a small landscape scale (Walker et al. 2005; Ishida et al. 2007). The structure of EMF communities in developed forests resembles those of highly diverse plant and animal communities (Magurran 2004).

To better understand EMF ecology and conservation efforts, it is crucial to explore how EMF communities develop into highly diverse communities. Such knowledge would also contribute to the conservation of entire forest ecosystems, because many tree species depend on nutrients supplied from EMF. However, minimal research has been conducted on the successional processes of EMF communities, because most studies have investigated EMF communities that have already developed (e.g., Gardes and Bruns 1996; Peter et al. 2001; Kennedy et al. 2003; Koide et al. 2005).

An additional area requiring further research concerns the functions of EMF in nature. It is widely accepted that EMF can significantly improve host growth by providing the host with mineral nutrients to which EMF have better access than plant roots alone. However, this role of EMF has mostly been demonstrated under simplified, artificial experimental conditions (Smith and Read 2008). Given the variable environmental conditions and complicated interorganismal interactions in the field, it is unwise to assume that EMF would perform these same functions in nature. In addition, the quantification of these functions and functional diversity would be challenging (Koide et al. 2007). For example, EMF are ubiquitous in forests, thus precluding adequate nonmycorrhizal control treatments that would be

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necessary for the accurate quantification of EMF contributions. As a result, investigations concerning the ecological functions of EMF have been impeded by such difficulties in controlling EMF in nature. These problems are common to most microbial experiments in the field (Copley 2000).

To remedy these complications of field research, one potential approach may be to study early successional sites in which EMF communities are in the process of developing. Some early successional sites consist of simple host communities established on relatively homogeneous substrates, allowing for a more thorough examination of EMF community development processes and the underlying mechanisms thereof. Moreover, primary successional sites offer one crucial advantage for the quantification of EMF functions in the field: there are no EMF propagules at the initial stage, thus permitting the establishment of nonmycorrhizal control treatments in potential EMF environments. By comparing EMF treatments with nonmycorrhizal treatments, the effects of EMF would be quantifiable in the field.

A variety of disturbances can create bare ground where a biological legacy is absent and primary succession begins (Walker and del Moral 2003). Such disturbances include volcanic activity, glacier retreat, sand dune movement, and mining. Specifically, intensive volcanic activity can abruptly create vast barren deserts completely covered by sterile substrates. These deserts are relatively large and homogeneous habitats relative to those created by other gradually occurring disturbances. Such homogeneous habitats would be ideal for testing fundamental ecological models that could be applied to other ecosystems. Indeed, many ecologists have been attracted to volcanic sites for these reasons (see Walker and del Moral 2003). Until recently, mycorrhizal fungi (especially EMF) have been ignored in studies of volcanic succession. However, data obtained from studies in volcanic systems may lead to a breakthrough in EMF ecology. In this chapter, I focus on EMF in volcanic deserts and use several basic ecological models to provide broadly applicable insights into EMF ecology.

2 Volcanoes: Unique Field Sites for EMF Studies

With the exception of the area at the center of an eruption, several preexisting organisms and organic materials often remain after an eruption. Therefore, it would be difficult to find truly primary successional areas with no biological legacy. Even if such areas were found, there is likely no general successional pattern common to all volcanic systems. In fact, a number of studies have demonstrated that several factors affect vegetation succession in volcanic areas, including environmental and geographical conditions, the type and magnitude of the eruption, ground substrates, and the surrounding vegetation (e.g., Walker and del Moral 2003). Thus, there is significant variation in the trajectory of plant succession, and the same may be true for EMF succession. However, a general pattern of EMF succession can be assumed: because most climax ecosystems are dominated by obligately mycorrhizal plants, these plants and mycorrhizal fungi must appear at some stage of succession and will eventually dominate.

EMF spores are relatively small and well-adapted to wind dispersal compared to arbuscular mycorrhizal fungi (AMF). These traits render EMF superior to AMF in reaching unexplored habitats. Although the data are limited, EMF have been found at sites in the early stages of primary volcanic succession. For example, Allen et al. (1992) noted that 5 years after the cataclysmic eruption, newly established conifers in a pumice plain of Mount St. Helens were colonized by EMF. In addition, *Salix* species (willows), which are known to be associated with EMF, are often the earliest colonizers after eruptions in cool climates (e.g., Mount Koma, Japan; Titus and Tsuyuzaki 2002). Recently, EMF on pioneer *Salix* have been intensively studied in a unique volcanic desert on Mount Fuji, Japan (Nara et al. 2003a, 2003b; Nara and Hogetsu 2004; Wu et al. 2005; Nara 2006a, 2006b; Wadud et al. 2006a, 2006b).

Mount Fuji, the highest mountain in Japan, erupted in 1707, and its southeastern area was completely covered with scoria up to 10 m deep. While all vegetation was initially destroyed, plant communities have been recovering. At present, more than 90% of the ground surface between 1,450 and 1,600 m asl is devoid of plant cover. Vegetation is now patchily distributed, forming isolated islands of various sizes in a sea of volcanic desert (Fig. 1). Such vegetation islands are often initially colonized by the nonmycorrhizal perennial herb *Polygonum cuspidatum*, which enlarges clonally to form a stable island within an unstable scoria desert (Hirose and Tateno 1984; Zhou et al. 2003). The stabilized habitat enables the further establishment of many plant species, including the pioneer ectomycorrhizal (EM) plant *Salix reinii* (hereafter *Salix*, unless otherwise specified). *Salix* is a ground-covering shrub species, usually less than 1 m in height. Approximately one-third of the vegetation islands

Fig. 1 A vegetation island in the volcanic desert on Mount Fuji, Japan. *Salix reinii (arrow)* is the pioneer ectomycorrhizal (EM) host species that recruits subsequent EM tree species, such as *Betula ermanii (arrowhead)* and *Larix kaempferi (double arrowhead)*

contain *Salix*. Other EM plants are quite rare (0.003% of the total *Salix* coverage). The coverage size and age of *Salix* within each vegetation island range from small (<0.1 m^2) and young (<5 years) saplings to large (>50 m^2) and old (>>100 years) shrubs (Nara et al. 2003a; Zhou et al. 2003). Therefore, this volcanic desert provides *various sizes* (or *various developmental stages*) of *a single host species* distributed in *isolated islands*, all of which are favorable conditions for ecological modeling.

3 Developmental Patterns of EMF Communities

Community developmental patterns can be assessed with several indices, including species richness (number of species), diversity (using diversity indices), community structure, and species turnover. Numerous observational and experimental studies have described patterns of plant and animal community development, resulting in many theoretical models of these easily observable organisms (e.g., Magurran 2004). In contrast, the developmental patterns of EMF communities have not been well studied, especially in primary successional sites.

A series of studies on secondary EMF succession demonstrated that EMF communities change with time and/or host growth (Last et al. 1984; Dighton et al. 1986). In many cases, "early-stage fungi" that initially colonize host plants are replaced by "late-stage fungi" that colonize plants later. Such secondary EMF succession is often initiated by propagules that have survived the disturbance (Horton et al. 1998; Baar et al. 1999) and mycelia spread from the remaining hosts (Perry et al. 1987). These studies also indicate that EMF community development is affected by both predisturbance communities and environmental heterogeneity. Thus, it may be difficult to observe and/or quantify general patterns in secondary successional sites. In contrast, primary succession begins on homogeneous sterile substrates; therefore, these sites may be more amenable to the observation of broadly applicable patterns. Fortunately, EMF data from the volcanic desert on Mount Fuji rival similar datasets from plant and animal community studies. Here, I applied several fundamental ecological models that have been developed for other well-studied organisms to the Mount Fuji EMF data.

3.1 Species Richness

Species richness is a simple and concise index used to describe ecological communities. This measure has been the most important criterion for conservation strategies and has thus been used in many ecological models (Magurran 2004). Species richness is also an important index for evaluating the developmental stages of communities. However, species richness is often difficult to measure properly, especially in diverse communities, because more species are recorded with an increase in sample size. In other words, sampling effort will affect the observed species richness Actually, even after an intensive soil sampling, it is impossible to detect all EMF species inhabiting a small forest (Horton and Bruns 2001; Taylor 2002).

The density of individuals at a site also affects species richness. Gotelli and Colwell (2001) clearly illustrated this point using an example of tree species in a tropical old-growth forest and a neighboring tropical second-growth forest. When the same number of quadrats was censused in both forests, more species were detected in the second-growth forest, despite higher species richness in the old-growth forest. This artifact occurred because stem density was higher (i.e., more smaller trees) in the second-growth forest and thus more individuals were included in the sampling. Similarly, EM root tip density often varies significantly among forests or even among plots within a forest, making it difficult to compare species richness across units of observation.

To overcome problems involving density and sampling effort in measuring species richness, species accumulation (or rarefaction) curves (SACs) can be an effective solution. EMF communities have often been examined using random soil sampling and molecular identification techniques (Horton and Bruns 2001). Therefore, it may appear reasonable to use samples on the x-axis in SACs to secure the independence of samples. However, the number of root tips in a soil sample often fluctuates considerably. Thus, even if sample-based SACs are used, the x-axis should be re-scaled to represent individual root tips (e.g., Fig. 2).

SACs of EMF were compared among the following host (*Salix*) developmental stages: small (<0.5 m^2 coverage area, <10 years old), medium (2–10 m^2, <50 years old),

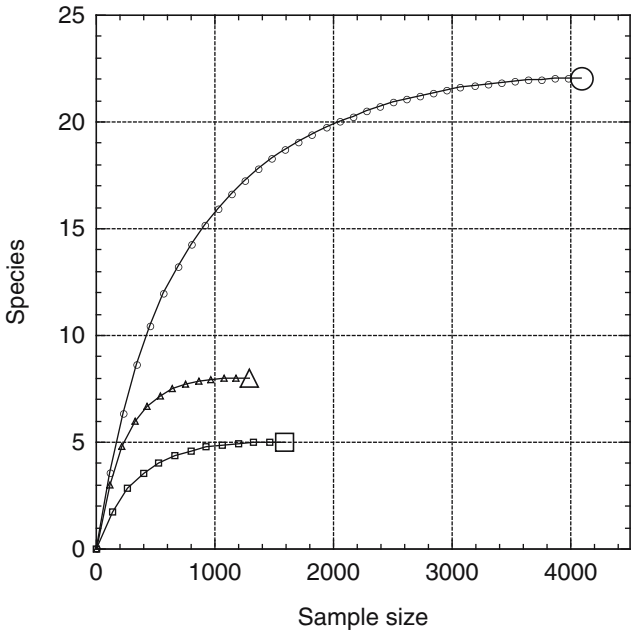

Fig. 2 Species accumulation curves for ectomycorrhizal (EM) fungal species in EM root tips collected from *Salix reinii* patches of different sizes: small (<0.5 m^2, square), medium (2–10 m^2, triangle), and large (>45 m^2, circle). Data for the large host size class include EM fungal species detected in three different types of spatial position. (Data from Nara *et al*. 2003b)

and large (>45 m^2, >>100 years old) shrubs (Fig. 2). Regardless of sampling effort or individual root tips examined, EMF richness was highest in large *Salix*, followed by medium and small *Salix* (Fig. 2). This pattern indicates that EMF species richness increases with the size of *Salix* patches (i.e., with host growth). In addition, the sampling effort needed to reach an asymptote of EMF richness increases with host growth stage. EMF richness is usually much higher in forest stands and does not reach an asymptote even after intensive sampling (Horton and Bruns 2001; Taylor 2002). EMF communities may gradually become species-rich with the succession of host vegetation.

3.2 Species–Area Relationships

One general and well-known relationship within ecology is the species–area relationship (SAR): large islands support more species than do small islands (Gotelli 2001). While SARs are often confused with SACs, the two concepts are fundamentally different (Rosenzweig 1995; Gray et al. 2004). Because of the potential importance of SARs for basic research as well as conservation efforts, they have been extensively studied within plant and animal communities (MacArthur and Wilson 1967; Connor and McCoy 1979; Rosenzweig 1995; Whittaker and Fernández-Palacios 2007). In contrast, SARs have been virtually ignored within fungal communities.

Data for constructing SARs are usually obtained from nested experimental designs or discrete island designs, in which several orders of area must be censused for species counts. While it is possible to census plant and animal species in these area ranges, such sampling efforts are quite challenging within fungal communities. Because SARs are greatly affected by the presence of rare species, these species must be detected even in the largest area sampled. EMF have been examined using random soil cores that represent less than 0.0001% of the ground surface in most studies. With this approach, we can not detect all rare species. But it is not realistic to excavate all root tips in a 100-m^2 area (or larger) and to identify all mycobionts using molecular identification. Species richness estimators may compensate in part for small sample sizes within a large area; however, a lack of consensus as to the best estimator index makes the application difficult. Using sequence similarity to define a "species" (or operational taxonomic unit) may also be problematic for SARs, because the threshold similarity used to group species would greatly affect the SAR (Green and Bohannan 2006).

In contrast to the molecular approach, the use of sporocarps allows for accurate EMF species definitions. Moreover, sporocarps correspond well to belowground EMF communities in the Mount Fuji desert, likely due to favorable conditions (e.g., abundant precipitation throughout the year) for sporocarp formation (Nara et al. 2003a, 2003b). In fact, more than 90% of belowground EM tips were dominated by EMF that produced sporocarps (Nara et al. 2003b). Weekly sporocarp census data were used in a power function SAR model, which is a common SAR model used to describe plant and animal communities:

$$S = cA^z \text{ (or } \log S = \log c + z \log A)$$

where S is the number of species in an given area (A), and c and z are constants determined from empirical data (Arrhenius 1921; Preston 1962). In log-log space, the intercept and slope are determined by c and z, respectively. In particular, z-values offer important information regarding spatial scale and conservation. The z-value for EMF in the Mount Fuji desert was 0.29 (Fig. 3), and the correlation was highly significant ($p < 0.001$). Although the limited spatial scale and the confounding effect of time may have affected the result, this z-value is within the normal range of z-values for plants and animals in island habitats ($z = 0.20$–0.35; MacArthur and Wilson 1967; Whittaker and Fernández-Palacios 2007).

Some researchers claim that microorganisms do not exhibit significant SARs (see Finlay 2002), but clearly the fungal SAR presented here (Fig. 3) is similar to those of plants and animals. This result is realistic, because fungi are multicellular eukaryotes that globally number 1.5 million expected species (Hawksworth 1991), a number comparable to those of the plant (0.3 million described species) and animal (1.3 million described species) kingdoms. There are several proposed mechanisms that may explain the SARs for plant and animal communities (see Whittaker and Fernández-Palacios 2007). These same mechanisms, particularly habitat diversity (described below), may also determine the SARs for fungal communities.

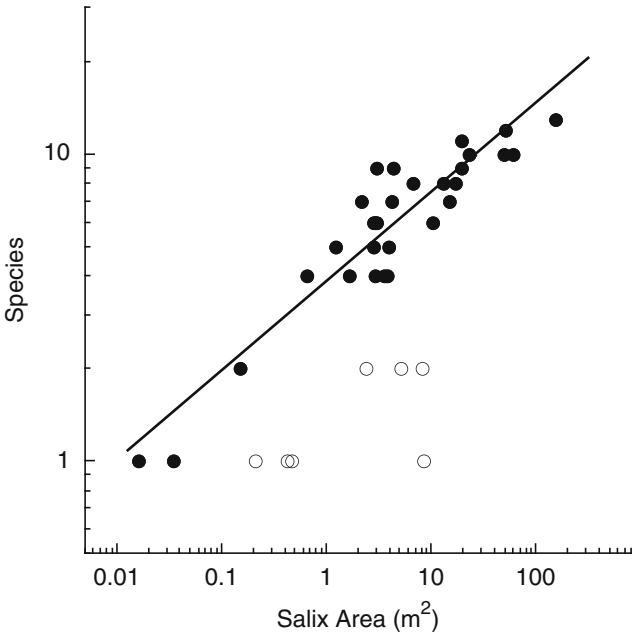

Fig. 3 Species–area relationships for ectomycorrhizal fungi (EMF). The number of EMF sporocarps recorded in each *Salix* island is plotted against the area of *Salix* size (data from Nara et al. 2003a). Due to poor photosynthetic abilities, some *Salix* produced few sporocarps (*open circles*). Although these less vigorous *Salix* were associated with a number of EMF species (5.5 ± 1.2 per *Salix*) in a belowground study (Nara et al. 2003b), they were excluded from the regression

3.3 Community Structure

Several ecological models have been developed to describe or predict community structure in nature (Tokeshi 1993). Species–rank abundance distributions (SADs) have often been used to graphically represent the structure of plant and animal (Magurran 2004) as well as EMF (Horton and Bruns 2001) communities. SADs are also effective for illustrating changes in structure during community development. Figure 4 presents SADs for EMF associated with small, medium, and large *Salix*, using belowground EM tips as the currency of relative abundance. The SADs clearly shifted from geometric series to log-normal relationships with the growth of *Salix*. The slope of the SADs significantly decreased with host size, indicating that species become relatively more even as hosts grow. A similar pattern was observed within secondary successional EMF communities after forest fires, in which EM morphotypes represented species (Visser 1995).

The geometric series model is based on the biological assumption that the most dominant species occupies a certain proportion of the limiting resource, with the second-most dominant species occupying the same proportion of the remaining

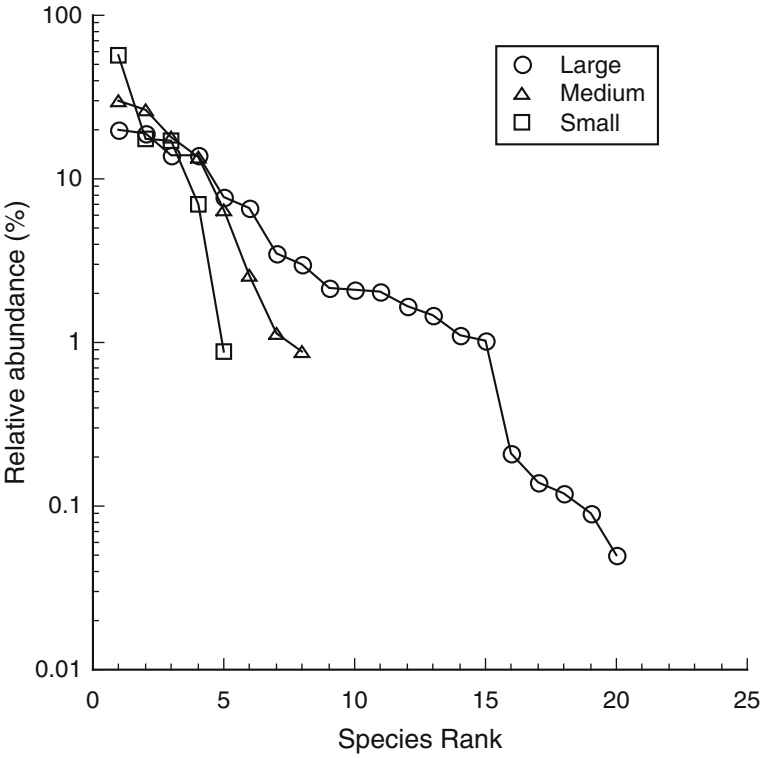

Fig. 4 Species–rank abundance distribution of ectomycorrhizal (EM) fungi, using the data of belowground EM tips collected from *Salix* of different sizes. Data from Nara et al. (2003b)

resource, and the third dominant species again using the same proportion of the remaining resource, until all species have been accommodated (Magurran 2004). This model fits data collected from sites that are species-poor due to disturbance, a harsh environment, or early successional stage (Tokeshi 1993, Magurran 2004). In contrast, species-rich undisturbed and developed communities appear to follow a log-normal pattern of relative species abundances (Preston 1962). In fact, the relative abundances of most organisms follow this pattern. The log-normal model was initially developed to obtain a better fit to field data and made no biological assumptions. However, the log-normal distribution is assumed to be a consequence of the central limit theorem: when a large number of factors acts to determine a variable in question (e.g., log-transformed abundance of each species), random variation in those factors will result in the variable being normally distributed (May 1975; Magurran 2004). This effect becomes more pronounced as the number of determining factors increases (Magurran 2004). Although the mechanisms underlying the shift from a geometric to a log-normal pattern remain uncertain, the number of determining factors is likely to increase as communities develop. The mechanisms that drive community development may be common to all organisms, including plants, animals, and fungi.

3.4 EMF Diversity

Both species richness and evenness (or relative abundance) affect the value of most diversity indices, including the well-known Shannon's (H) and Simpson's (1/D) indices. As with species richness, sampling effort must be considered when diversity indices are compared among different communities (Fig. 5). Shannon's index approached an asymptote in each EMF community from small, medium, and large *Salix* in the volcanic desert on Mount Fuji (Fig. 5a). Regardless of sampling effort, Shannon's index was highest in large *Salix*, followed by medium and small *Salix*. Using Simpson's index, sampling effort in medium *Salix* appeared to be insufficient for reaching an asymptote (Fig. 5b). However, this index is often robust against insufficient sampling when comparing communities (Magurran 2004). When rarefied to any sample size, the order of Simpson's diversity was constant: large > medium > small. Both diversity indices clearly indicated that the EMF community became more diverse as host size increased.

It is difficult to compare diversity indices among studies, because sampling effort and strategy vary considerably. Despite this, EMF diversity indices in most forest stands are considerably higher than those for EMF communities in the volcanic desert. For example, Simpson's index was 60.1 in a secondary conifer-broadleaf mixed forest, Japan (Ishida et al. 2007), which was about seven times larger than that of EMF in the volcanic desert. As predicted in the SAR model (see above), habitat size was positively correlated with species richness. The SADs presented here indicate that species evenness increased as host size increased. Because both species richness and evenness increased with host size, diversity should also

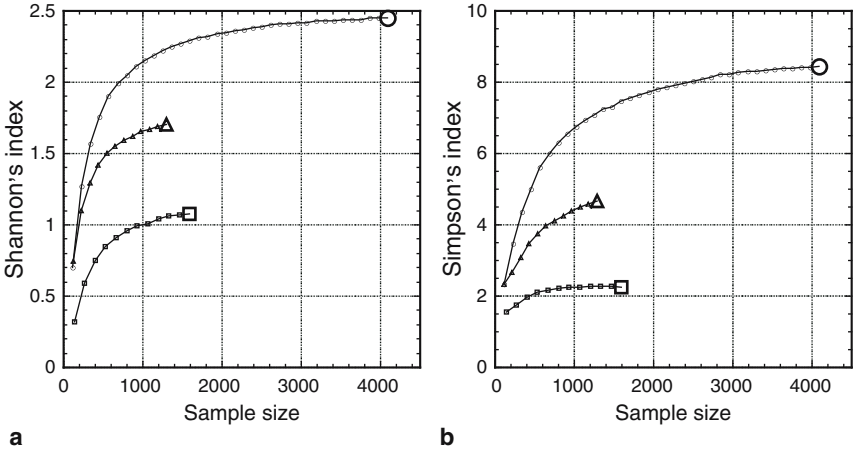

Fig. 5 Increase in ectomycorrhizal fungal diversity with host plant development, taking into account sampling effort. **a** Shannon's H, **b** Simpson's 1/D. Data from belowground EM tips were used (Nara et al. 2003b)

increase until the entire ground surface is covered with host plants. Today, 300 years after the last eruption, only approximately 1% of the ground surface of the Mount Fuji site is covered with EMF plants. It may take more than 1,000 years for the volcanic desert to become a closed forest of EMF trees (similar to sites at the same elevation on other faces of Mount Fuji) that could accommodate a diverse EMF community.

3.5 Primary Succession of Ectomycorrhizal Fungi

In all the above models using SACs, SARs, SADs, and diversity indices, species must be distinguished from one another. Once species are defined, however, any label can be assigned or exchanged without affecting model results. Such interchangeability indicates that species identity is not considered in these analyses. Because the species pool differs significantly among locations, species identity may not have broader implications. However, together with information on ecological traits, species identity can provide more general insight. Below, I address the observed sere of EMF species and their associated ecological traits.

Aboveground sporocarp and belowground EMF communities in the volcanic desert exhibited a clear successional pattern corresponding to the developmental stages of *Salix* (Fig. 6; see also Nara et al. 2003a, 2003b). Newly-established *Salix* seedlings in a nonmycorrhizal vegetation island were colonized by one of three first-stage EMF: *Inocybe lacera, Laccaria amethystina*, or *Laccaria laccata*. These species shared several traits of first colonizers. For example, in plant succession, early colonizers are often ruderals that produce abundant seeds in short rotations and adapt

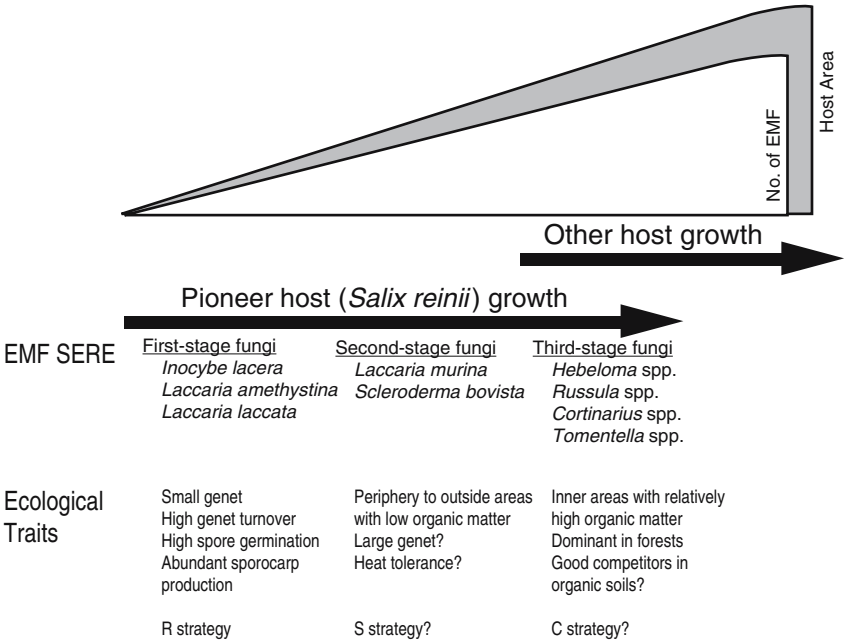

Fig. 6 The successional pattern of ectomycorrhizal fungi in the volcanic desert on Mount Fuji, Japan, noting the ecological traits

to open environments. Similarly, all first-stage fungi produce abundant sporocarps in the Mount Fuji desert (Nara et al. 2003a). They can also produce sporocarps even in association with current-year seedlings in an inoculation experiment (Nara, unpublished data). Moreover, the spore germination rates of the first-stage fungi were among the highest ever recorded for EMF (up to 68% in the presence of host roots; Ishida et al., submitted). Genet analyses of both *Laccaria* species at this site showed that the populations were exclusively dominated by small genets and exhibited a high rate of genet turnover (Wadud 2007), which are both indicative of spore-dependent regeneration. Therefore, such ecological (ruderal/R-strategy) traits of the first-stage EMF may enable these species to be early colonizers of new habitat.

As the host *Salix* grew, two later-colonizing species, *Laccaria murina* and *Scleroderma bovista*, were often observed (Nara et al. 2003a, 2003b). These species usually appeared on the edges and outside of each vegetation island, indicating tolerance to soil stresses such as high temperature, physical disturbance, and low organic substrates. Previous research demonstrated that one *Scleroderma* species is tolerant to high temperatures (Ingleby et al. 1985). Thus, these later-colonizing species may be similar to stress-tolerator (S strategy) plants.

Further host development fostered the colonization of *Hebeloma, Cortinarius, Russula*, and Thelephoraceae species, primarily inside the vegetation islands (Nara et al. 2003a, 2003b). *Hebeloma* species are early colonizers in some secondary successional sites (e.g., Last et al. 1984) and often reproduce largely by spore

regeneration (Gryta et al. 1997). Similarly, all *Hebeloma* species in the Mount Fuji volcanic desert exhibited high rates of spore germination that were similar to those of first-stage EMF (Ishida et al., submitted). In general, the soil at secondary successional sites is more developed relative to primary successional sites. *Hebeloma* species tend to be ruderal in relatively developed soil but are unable to colonize extremely undeveloped soils. Data concerning the ecological traits of *Cortinarius, Russula*, and Thelephoraceae species are scarce, but species belonging to these taxa usually dominate many forest ecosystems, in which the soil often contains considerable organic substrates. In the Mount Fuji volcanic desert, these species were only observed within large *Salix* islands, where the soil was relatively developed. Thus, these EMF species may have better competitive abilities in organic soils, similar to competitor (C-strategy) plant species.

Ascomycetes were minor EMF components in this early successional desert (Nara et al. 2003b). The predominant vegetative form of ascomycetes is haploid; thus, a single spore can establish a vegetative form, which would seemingly be a great advantage for colonizing open areas. However, the Mount Fuji desert was instead dominated by Basidiomycete species, most of which need two compatible spores for the establishment of a functional vegetative form. Such a requirement could be a strong disadvantage for colonizing large open sites, but the basidiomycete spore dispersal mechanism is well adapted for wind dispersal, perhaps overcoming this disadvantage.

One ascomycete, the cosmopolitan *Cenococcum geophilum*, was found in 22 of 311 soil samples (Wu et al. 2005). At this site, the populations of *C. geophilum* were dominated by a few large genets in contrast to the high genet diversity in relatively developed sites. This low genet diversity may indicate a limited invasion ability from outside of the vegetation islands. This species forms abundant sclerotia but no ascospores. Sclerotia may not be suitable for colonizing large open sites, because they are much larger than spores.

Although it is difficult to construct a broadly applicable model of EMF succession so far, the ecological traits addressed above should be considered in future ecological modeling of EMF succession or community development.

4 Ecological Role of EMF

Unequivocal evidence of the importance of EMF in ecological processes is surprisingly scarce. This lack of data is largely a result of the ubiquity of EMF and the consequent inability to establish nonmycorrhizal controls in nature. Volcanic areas are one of the rare natural locations where nonmycorrhizal controls are feasible, providing a unique opportunity to experimentally quantify EMF functions in the field. Below, I summarize data related to the ecological role of EMF in volcanic deserts. Horton and van der Heijden (2007) provided a concise review of the effects of EMF on seedling establishment in other environments.

4.1 EMF Effects on Seedling Establishment

Volcanic substrates are extremely unbalanced in the nutrients required for plant growth and are particularly deficient in nitrogen (N). EMF can use a wider range of N sources relative to nonmycorrhizal roots and AMF (e.g., Abuzinadah and Read 1986). A substantial portion of the N absorbed by EMF is subsequently transferred to host plants (Arnebrant et al. 1993; He et al. 2003). Most pioneer EMF host plants are anemochoric, and their tiny seeds contain low amounts of N. Thus, without N supplied by EMF, pioneer plants may not survive the early establishment processes. Therefore, EMF may potentially contribute to plant establishment even during early successional stages when plant growth is often severely N-limited.

To initially exploit nonmycorrhizal areas, spores must be the dominant form of EMF sources. However, dormant spores are rare in the Mount Fuji volcanic desert, even though the total amount of dispersed spores was likely quite large during the 300 years post-eruption (Nara and Hogetsu 2004). In addition, current-year spores are often dispersed during the late growing season (Fig. 7). Thus, current-year seedlings may have difficulty accessing compatible spores in the early growing season and may not reach a size guaranteeing their survival during the first winter. Consequently, initial plant establishment in nonmycorrhizal habitats appears to be quite difficult, as evidenced by the 0.025 events/ha/year (calculated from 161 *Salix* islands in 21 ha during the 300 years since the last eruption; Nara 2006b). In other modes of succession, the initial EM establishment appears to be relatively easy due to dormant spores (Horton et al. 1998, Baar et al. 1999; Jumpponen 2003; Ashkannejhad and Horton 2006). Although the mechanisms behind initial EM establishment in nonmycorrhizal

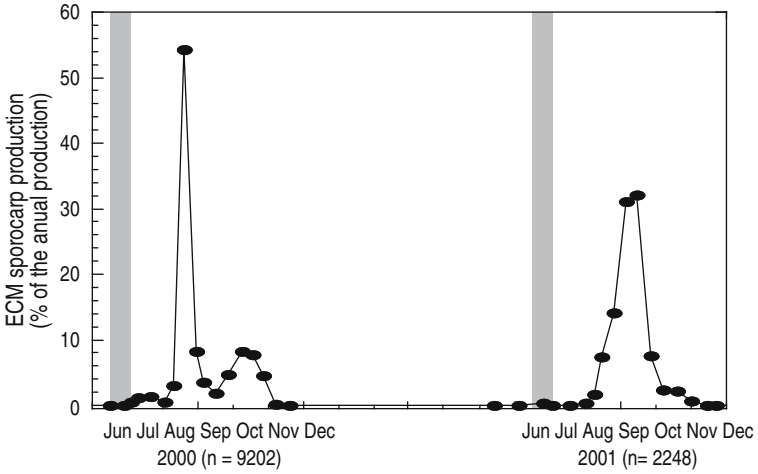

Fig. 7 Seasonality of sporocarp formation in the volcanic desert on Mount Fuji, Japan. *n* Number of sporocarps produced each year. *Gray zones* indicate seasons of *Salix reinii* seed dispersal. This pioneer EM host plant usually germinates immediately after the seed has landed

habitats remain unknown for volcanic settings, several extremely rare and serendipitous events may be important. For example, small nutrient-rich microhabitats provided by insects and animals may enable current-year seedlings to survive until the arrival of compatible spores dispersed during the sporocarp season.

In the Mount Fuji desert, the initial establishment of an EMF plant in a nonmycorrhizal habitat facilitates the subsequent establishment of conspecific seedlings in the vicinity (Lian et al. 2003). This facilitation is largely due to more readily available EMF sources (e.g., vegetative EMF mycelia from the established *Salix*) early in the growing season. In a field transplant experiment, *Salix* seedlings were easily colonized by EMF in the vicinity of naturally established shrubs (27/33 seedlings were colonized) but were rarely colonized in the absence of shrubs (1/31 seedlings were colonized; Nara and Hogetsu 2004). A second transplant experiment, in which all biotic and abiotic conditions were identical except for the presence or absence of EMF mycelia, demonstrated that the improved seedling performance near established trees was solely attributable to EMF infection by extramatrical mycelia (Fig. 8; Nara 2006a).

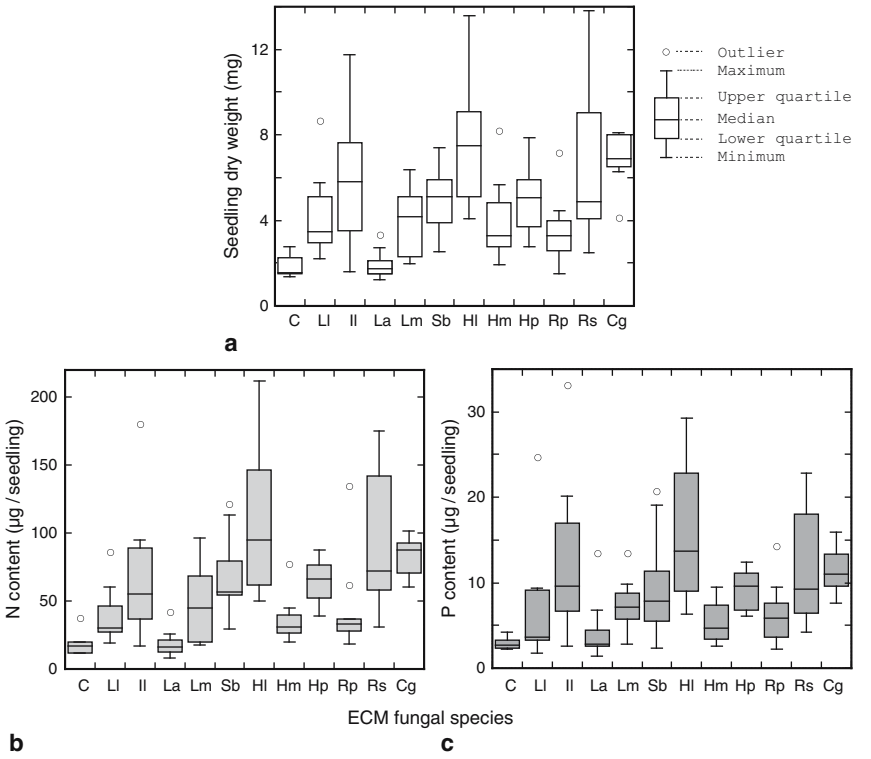

Fig. 8 Effects of common mycorrhizal networks of individual ectomycorrhizal fungal species on **a** dry weight, **b** nitrogen content, and **c** phosphorus content of current-year *Salix reinii* seedlings in an early-successional volcanic desert on Mount Fuji. *C* Control, *Ll Laccaria laccata, Il Inocybe lacera, La Laccaria amethystina, Lm Laccaria murina, Sb Scleroderma bovista, Hl Hebeloma leucosarx, Hm Hebeloma mesophaeum, Hp Hebeloma pusillum, Rp Russula pectinatoides, Rs Russula sororia, Cg Cenococcum geophilum*

Without such EMF mycelial systems in unmanipulated treatments, nearly all germinating seedlings die within 1 month. Therefore, the EMF mycelial systems (i.e., the mycorrhizal network) between established trees and seedlings play a critical role in conspecific seedling establishment. Although the importance of EMF networks for seedling establishment has been addressed in several studies (Perry et al. 1987; Horton et al. 1999; Dickie et al. 2002; Simard and Durall 2004), strong conclusive evidence was confounded by unavoidable infections from other sources both in control and experimental treatments. Results from the Mount Fuji primary successional desert clearly confirm the importance of EMF networks in seedling establishment.

The dominance of nonmycorrhizal conditions in the desert permitted the establishment of field mycorrhizal treatments, consequently facilitating the quantification of EMF functions and functional diversity in the field (Nara 2006a). Most fungal species, including the rarely studied *Inocybe* and *Russula* species, were isolated from the Mount Fuji desert, and the functions of their mycelial networks were examined. With the exception of one fungal species, EMF networks significantly improved seedling performance relative to nonmycorrhizal treatments. Nitrogen and phosphorus contents of the largest seedling in fungal treatments were five and eight times higher, respectively, than the maximum N and P content of the control seedlings (Fig. 8; Nara 2006a). These results highlight several broader implications of the importance of EMF communities. Without EMF networks, small seedlings would be unable to acquire N and P while competing with larger established plants. Moreover, the relationship between EMF networks of individual fungal species and plant seedlings is mostly mutualistic.

4.2 EMF Effects on Plant Succession

The EMF community on *Salix* was dominated by generalist species that were able to infect a wide range of host species in the Mount Fuji desert. For example, *Betula ermanii* (Betulaceae) and *Larix kaempferi* (Pinaceae) transplanted near the established *Salix* shrubs were colonized almost exclusively by EMF common to *Salix* (Nara and Hogetsu 2004). In addition, EMF found on *Salix* dominated naturally established *Betula* and *Larix* individuals, most of which were more than 10 years old (Nara 2006b). Surprisingly, all naturally established individuals (39 *Betula* and 26 *Larix*) were only observed in the vicinity of early-established *Salix* shrubs. The importance of *Salix* was evident given the probability ($p = 2.9 \times 10^{-12}$) of this establishment pattern under the null hypothesis that *Betula* and *Larix* could establish regardless of *Salix*. These results clearly indicate that both tree species were unable to establish in the absence of EMF networks. The establishment of tree species is a critical step in vegetation succession toward forest formation. Therefore, EMF networks may indeed drive vegetation succession.

EMF networks also serve an important role in ecological processes after the formation of forests, which comprise many EMF trees and EMF-dependent plants. In undisturbed forests, regenerating tree seedlings are quickly colonized by EMF

common to adult trees (Jonnson et al. 1999, Matsuda and Hijii 2004). Supported by abundant carbohydrates from adult trees, EMF networks are vastly superior to spores in colonizing seedlings that cannot spare much carbohydrate for EMF. Thus, EMF networks are likely to be the dominant form of infection for regenerating seedlings. After forest clear-cuts, understory shrubs act as refugia for EMF and facilitate subsequent seedling establishment of tree species (e.g., Perry et al. 1987). Therefore, EMF networks can function as a resilience mechanism for forest ecosystems.

Relative to EMF networks, the contribution of AMF networks is less clear. In volcanic sites, established AM plant patches do not improve subsequent plant establishment (Titus and del Moral 1998), due in part to the limited ability of AMF to improve plant N acquisition in N-poor volcanic substrates (Fujiyoshi et al. 2006). Even in developed soils, AMF networks do not improve plant performance (Kytoviita et al. 2003). AMF colonization is undoubtedly crucial to plant growth in many AMF ecosystems, but this is not necessarily due to a fungal network. Spores can be potentially substituted for a network in AMF ecosystems, because AMF spores are large (up to >500 μm in diameter) and contain more carbon resources than EMF spores (at most ca. 10 μm along the major axis). AMF mycelial systems are less differentiated compared to the highly differentiated mycelial systems of EMF. These differences in the mycelial systems may lead to different patterns of within-network nutrient movement between AMF and EMF, subsequently causing differences in seedling performance within the network. Although more research is necessary, the relative contribution of networks to ecological processes is likely greater in EMF than AMF.

In plant successional models, soil nutrient forms determine the shifts in types of mycorrhizae: AM plant communities are gradually replaced by EM forests with organic components becoming dominant in the soil (Smith and Read 2008). Given the superior ability of EMF to utilize organic materials (Abuzinadah and Read 1986), organic soils are certainly favorable to EMF in most environments. However, organic soil development is unlikely to be the only mechanism leading to the succession of mycorrhizal types. As described above, EMF networks are effective in recruiting subsequent EM plants, ensuring the successful regeneration of EM trees and improving the resilience of forest ecosystems. Therefore, once EMF networks have established, EM systems are likely to develop and may prevent themselves from following an alternative successional trajectory. Thus, EMF network would be another determining mechanism in plant succession.

References

Abuzinadah RA, Read DJ (1986) The role of proteins in the nitrogen nutrition of ectomycorrhizal plants .1. utilization of peptides and proteins by ectomycorrhizal fungi. New Phytol 103:481–493

Allen MF, Crisafulli C, Friese CF, Jeakins SL (1992) Re-formation of mycorrhizal symbioses on Mount St Helens, 1980–1990 – interactions of rodents and mycorrhizal fungi. Mycol Res 96:447–453

Arnebrant K, Ek H, Finlay RD, Soderstrom B (1993) Nitrogen translocation between *Alnus glutinosa* (L) Gaertn seedlings inoculated with *Frankia* sp and *Pinus contorta* Doug Ex-Loud seedlings connected by a common ectomycorrhizal mycelium. New Phytol l24:231–242

Arrhenius O (1921) Species and area. J Ecol 9:95–99

Ashkannejhad S, Horton TR (2006) Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving *Pinus contorta*, suilloid fungi and deer. New Phytol 169:345–354

Baar J, Horton TR, Kretzer AM, Bruns TD (1999) Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand-replacing wildfire. New Phytol 143:409–418

Connor EF, McCoy ED (1979) Statistics and biology of the species–area relationship. Am Nat 113:791–833

Copley J (2000) Ecology goes underground. Nature 406:452–454.

Dickie IA, Koide RT, Steiner KC (2002) Influences of established trees on mycorrhizas, nutrition, and growth of *Quercus rubra* seedlings. Ecol Monogr 72:505–521

Dighton J, Poskitt JM, Howard DM (1986) Changes in occurrence of basidiomycete fruit bodies during forest stand development – with specific reference to mycorrhizal species. Trans Br Mycol Soc 87:163–171

Finlay BJ (2002) Global dispersal of free-living microbial eukaryote species. Science 296:1061–1063

Fujiyoshi M, Kagawa A, Nakatsubo T, Masuzawa T (2006) Effects of arbuscular mycorrhizal fungi and soil developmental stages on herbaceous plants growing in the early stage of primary succession on Mount Fuji. Ecol Res 21:278–284

Gardes M, Bruns TD (1996) Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Can J Bot 74:1572–1583

Gotelli NJ (2001) A primer of ecology, 3rd edn. Sinauer, Sunderland, Mass.

Gotelli NJ, Colwell RK (2001) Quantifying biodiversity: procedures and pitfalls in the measurement and comparison of species richness. Ecol Lett 4:379–391

Gray JS, Ugland KI, Lambshead J (2004) On species accumulation and species–area curves. Global Ecol and Biogeol 13:567–568

Green J, Bohannan BJM (2006) Spatial scaling of microbial biodiversity. Trends Ecol Evol 21:501–507

Gryta H, Debaud JC, Effosse A, Gay G, Marmeisse R (1997) Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum* in coastal sand dune forest ecosystems. Mol Ecol 6:353–364

Hawksworth DL (1991) The fungal dimension of biodiversity – magnitude, significance, and conservation. Mycol Res 95:641–655

He XH, Critchley C, Bledsoe C (2003) Nitrogen transfer within and between plants through common mycorrhizal networks (CMNs). Crit Rev Plant Sci 22:531–567

Hirose T, Tateno M (1984) Soil nitrogen patterns induced by colonization of *Polygonum cuspidatum* on Mt Fuji. Oecologia 61:218–223

Horton TR, Bruns TD (2001) The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. Mol Ecol 10:1855–1871

Horton TR, van der Hijden MGA (2007) The role of symbioses in seedling establishment and survival. In: Leck MA, Parker VT, Simpson RL (eds) Seedling ecology and evolution. Cambridge University Press, Cambridge

Horton TR, Cázares E, Bruns TD (1998) Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. Mycorrhiza 8:11–18

Horton TR, Bruns TD, Parker VT (1999) Ectomycorrhizal fungi associated with *Arctostaphylos* contribute to *Pseudotsuga menziesii* establishment. Can J Bot 77:93–102

Ingleby K, Last FT, Mason PA (1985) Vertical-distribution and temperature relations of sheathing mycorrhizas of *Betula* spp growing on coal spoil. For Ecol Manage 12:279–285

Ishida TA, Nara K, Hogetsu T (2007) Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer–broadleaf forests. New Phytol 174:430–440

Jumpponen A (2003) Soil fungal community assembly in a primary successional glacier forefront ecosystem as inferred from rDNA sequence analyses. New Phytol 158:569–578
Kennedy PG, Izzo AD, Bruns TD (2003) There is high potential for the formation of common mycorrhizal networks between understorey and canopy trees in a mixed evergreen forest. J Ecol 91:1071–1080
Koide RT, Xu B, Sharda J (2005) Contrasting below-ground views of an ectomycorrhizal fungal community. New Phytol 166:251–262
Koide RT, Courty PE, Garbaye J (2007) Research perspectives on functional diversity in ectomycorrhizal fungi. New Phytol 174:240–243
Kytoviita MM, Vestberg M, Tuom J (2003) A test of mutual aid in common mycorrhizal networks: established vegetation negates benefit in seedlings. Ecol 84:898–906
Last FT, Mason PA, Ingleby K, Fleming LV (1984) Succession of fruitbodies of sheathing mycorrhizal fungi associated with *Betula pendula*. For Ecol Manage 9:229–234
Lian CL, Oishi R, Miyashita N, Nara K, Nakaya H, Wu BY, Zhou ZH, Hogetsu T (2003) Genetic structure and reproduction dynamics of *Salix reinii* during primary succession on Mount Fuji, as revealed by nuclear and chloroplast microsatellite analysis. Mol Ecol l12:609–618
MacArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton University Press, Princeton, N.J.
Magurran AE (2004) Measuring biological diversity. Blackwell, Malden, Mass.
Matsuda Y, Hijii N (2004) Ectomycorrhizal fungal communities in an *Abies firma* forest, with special reference to ectomycorrhizal associations between seedlings and mature trees. Can J Bot 82:822–829
May RM (1975) Patterns of species abundance and diversity. In: Cody ML, Diamond JM, eds. Ecology and evolution of communities. Harvard University Press, Cambridge, Mass., pp 81–120
Nara K (2006a) Ectomycorrhizal networks and seedling establishment during early primary succession. New Phytol 169:169–178
Nara K (2006b) Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert. New Phytol 171:187–198
Nara K, Hogetsu T (2004) Ectomycorrhizal fungi on established shrubs facilitate subsequent seedling establishment of successional plant species. Ecology 85:1700–1707
Nara K, Nakaya H, Hogetsu T (2003a) Ectomycorrhizal sporocarp succession and production during early primary succession on Mount Fuji. New Phytol l58:193–206
Nara K, Nakaya H, Wu BY, Zhou ZH, Hogetsu T (2003b) Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. New Phytol 159:743–756
Perry DA, Molina R, Amaranthus MP (1987) Mycorrhizae, mycorrhizospheres, and reforestation – current knowledge and research needs. Can J For Res 17:929–940
Peter M, Ayer F, Egli S, Honegger R (2001) Above- and below-ground community structure of ectomycorrhizal fungi in three Norway spruce (*Picea abies*) stands in Switzerland. Can J Bot 79:1134–1151
Preston FW (1962) Canonical distribution of commonness and rarity. Ecology 43:185–215, 410–432
Rosenzweig ML (1995) Species diversity in space and time. Cambridge University Press, Cambridge
Simard SW, Durall DM (2004) Mycorrhizal networks: a review of their extent, function, and importance. Can J Bot 82:1140–1165
Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, London
Taylor AFS (2002) Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant Soil 244:19–28
Titus JH, del Moral R (1998) The role of mycorrhizal fungi and microsites in primary succession on Mount St. Helens. Am J Bot 85:370–375
Titus JH, Tsuyuzaki S (2002) Arbuscular mycorrhizal distribution in relation to microsites on recent volcanic substrates of Mt. Koma, Hokkaido, Japan. Mycorrhiza 12:271–275

Tokeshi M (1993) Species abundance patterns and community structure. In: Advances in ecological research, vol 24. Academic, London, pp 111–186

Visser S (1995) Ectomycorrhizal fungal succession in Jack Pine stands following wildfire. New Phytol 129:389–401

Wadud MA (2007) Reproduction ecology of pioneer ectomycorrhizal fungi, *Laccaria amethystina* and *L. laccata*, in the volcanic desert on Mount Fuji. PhD thesis, The University of Tokyo.

Wadud MA, Lian CL, Nara K, Ishida TA, Hogetsu T (2006a) Development of microsatellite markers from an ectomycorrhizal fungus, *Laccaria amethystina*, by a dual-suppression-PCR technique. Mol Ecol Notes 6:130–132

Wadud MA, Lian CL, Nara K, Hogetsu T (2006b) Isolation and characterization of five microsatellite loci in an ectomycorrhizal fungus *Laccaria laccata*. Mol Ecol Notes 6:700–702

Walker JF, Miller OK, Horton JL (2005) Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. Mol Ecol 14:829–838

Walker LR, del Moral R (2003) Primary succession and ecosystem rehabilitation. Cambridge University Press, Cambridge

Whittaker RJ, Fernández-Palacios JM (2007) Island biogeography: ecology, evolution, and conservation. Oxford University Press, New York

Wu BY, Nara K, Hogetsu T (2005) Genetic structure of *Cenococcum geophilum* populations in primary successional volcanic deserts on Mount Fuji as revealed by microsatellite markers. New Phytol 165:285–293

Zhou ZH, Miwa M, Nara K, Wu BY, Nakaya H, Lian CL, Miyashita N, Oishi R, Maruta E, Hogetsu T (2003) Patch establishment and development of a clonal plant, *Polygonum cuspidatum*, on Mount Fuji. Mol Ecol 12:1361–1373

Colonization of Plant Roots by Pseudomonads and AM Fungi: A Dynamic Phenomenon, Affecting Plant Growth and Health

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1 Introduction

Because of their enormously large range of plant hosts and role in plant nutrition, arbuscular mycorrhizal (AM) fungi represent an extraordinarily fascinating field of study. Plant growth promotion effects by AM fungi were described as early as 1900 (Sthal 1900) and several data obtained in the second half of the last century support the idea that these microrganisms can act as biocontrol agents (BCA).

The extent of root colonization is variable in different plants and under different environmental conditions (Giovannetti and Hepper 1985). Some effects of AM colonization on plants have been reported to be dependent on the degree of root colonization, while others have not. Root exudation and pH are modified by the presence of AM fungi (Bansal and Mukerji 1994; Bago et al. 1996), therefore AM fungi can affect the growth of rhizobacteria. Similarly, both root colonization by AM fungi and their effects on the plant can be affected by the presence of rhizobacteria, which can be plant growth-promoting, mycorrhiza helper or biocontrol agents.

Although several genera of microorganisms have been reported to behave as biocontrol agents (BCA) or plant growth-promoting rhizobacteria (PGPR), over these years attention has been focused mainly on fluorescent pseudomonads, because they are common inhabitants of rhizosphere and phyllosphere environments, synthesize a wide range of metabolites and enzymes (O'Sullivan and O'Gara 1992), are easily isolated from natural environments, utilize a large variety of substrates (Latour and Lemanceau 1997), and are easy to culture and manipulate genetically (Walsh et al. 2001). Altogether, these characters make them more reliable to scientific experimentation (Whipps 1997; Haas and Défago 2005). Due to their impressive capacity in producing different types of antibiotics such as 2,4-diacetylphloroglucinol, phenazines, oomycin, pyoluteorin, pyrrolnitrin,

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viscosinamide (Raaijmakers et al. 2002) and some antifungal factors (i.e., lytic enzymes), fluorescent pseudomonads are thought to represent a valid alternative to the use of chemical pesticides to protect plants against soilborne diseases. Although fluorescent pseudomonads under controlled conditions demonstrate high potential for their exploitation in applicative process, frequent inconsistent field-performances, related to low survival, have been reported (Schippers et al. 1987; Weller 1988). Their efficiency is indeed strongly dose-dependent (Bull et al. 1991; Raaijmakers et al. 1995). The presence of the PGPR/BCA strains and their phytostimulating/antifungal factors at the right moment (i.e., time of attack by a phytopathogen) and root location (i.e., penetration site of the phytopathogen) is unquestionable.

2 Plant Root Colonization by Bacterial Cells

Root colonization is the process by which bacteria introduced on seeds, vegetatively propagated plant parts, or into soil become distributed along roots (Weller and Thomashow 1994). Plant-associated bacteria could establish a more or less intimate relationship with the root system depending on their preferential localization inside (endophytic bacteria) or outside the plant tissue, both on the root surface (rhizoplane) and on the volume of soil surrounding the root system (rhizosphere).

The root colonization process is promoted by the release of a wide range of low and high molecular weight molecules by the plant root in the rhizosphere (Bais et al. 2006). Root exudation is a cost to the plant and it has been calculated that an average of 17% of the net fixed carbon is released by the roots (Nguyen 2003). The amount of photosynthates released by the root system is dependent on the plant age and physiological state, the substrate in which the plant is grown and the bioavailability of nutrients (Walker et al. 2003). The release of those exudates leads to the enhancement of specific soilborne populations which are adapted to the use of the corresponding organic compounds for their metabolism. Indeed, rhizospheric populations of fluorescent pseudomonads were shown to have a carbon and energetic metabolism differing from those from bulk soils (Latour and Lemanceau 1997). More specifically, they have the ability to use specific organic compounds of root exudates giving them a competitive advantage over soil bacteria which do not have this ability. By following the chemoattractant effect, bacteria “sense” and move towards the root and settle where the root exudates are released at a higher rate. In these specific sites, single bacterial cells bind to the root (Fig. 1a) and start to replicate originating little clusters (Fig. 1b), elongated (Fig. 1c, d) or thick (Fig. 1e) microcolonies and, finally, true biofilm embedded in an extracellular matrix (Fujishige et al. 2006) in which cell activity is regulated by quorum-sensing (Watnick and Kolter 2000). Although the patterns of microbial root colonization differ among plant species, the percentage of the root surface covered by bacteria has been calculated to be less than 10%. The bacterial cell distribution is often

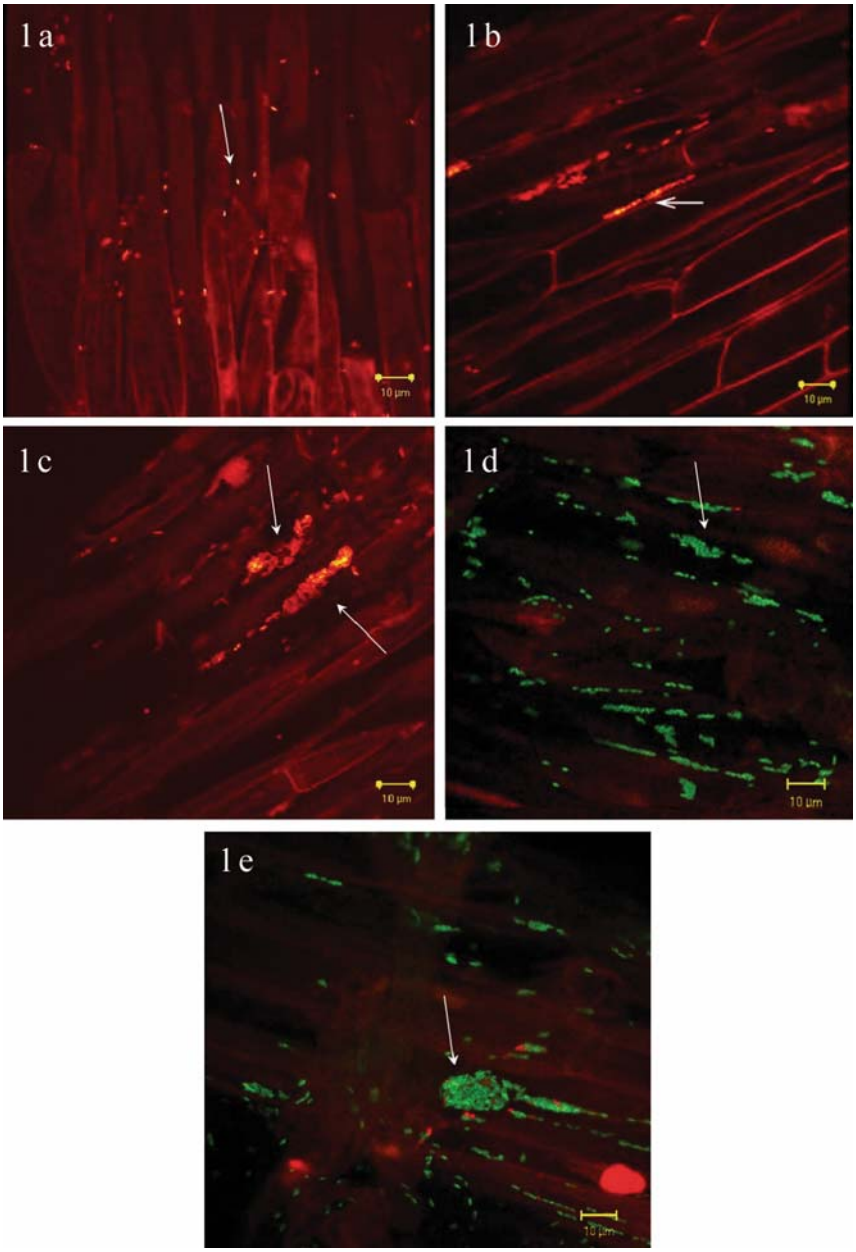

Fig. 1 Root colonization pattern of *Pseudomonas fluorescens* along tomato root: single bacterial cells on the elongation zone (Fig. 1**a**), little clusters of cells (Fig. 1**b**), and elongated (Fig. 1**c–d**) or thick (Fig. 1**e**) microcolonies

heterogeneous, with heavily colonized sites alternated to zones without detectable colonization (Chin-A-Woeng et al. 1997).

3 Dynamic of the Spatio-Temporal Pattern of Root Colonization by Fluorescent Pseudomonads

Root colonization by a beneficial strain is a dynamic spatio-temporal process, depending on the age of the plant and on the root zones, strictly related to the root growth, development and differentiation (Baudoin et al. 2001). The first requirement for a PGPR or BCA strain is the capability to establish and maintain threshold population densities on the root (Bull et al. 1991; Raaijmakers et al. 1995). However, after inoculation, the density of the introduced bacterial strain usually declines to a level depending on its rhizospheric competence, the host plant and the resident microflora (Weller 1983; Weller and Thomashow 1994; van Veen et al. 1997). Reductions of the density of fluorescent pseudomonads on the whole root system have been reported several times. As an example, the cell density of the PGPR and Mycorrhiza Helper Bacteria (MHB) *Pseudomonas fluorescens* 92rk in the tomato rhizosphere decreased from 2.34×10^8 down to 6.16×10^5 cfu/g of root 28 days after inoculation (Gamalero et al. 2004b). Both biotic and abiotic factors can negatively affect the survival of the introduced strain (Strigul and Kravchenko 2006). A significant effect of soil fertility (abiotic factor), on the survival of the strain *P. fluorescens* A6RI in the rhizosphere of tomato plant, has been reported by Gamalero et al. (2002), as densities of the strain A6RI were significantly higher in the rhizosphere of tomato grown in a 1:2 than in a 2:1 soil:sand mixture. Under the low fertility condition, the bacterial density increased during the first week and decreased the two following weeks to finally reach a plateau (3.16×10^6 cfu/g root) while, in the high fertility condition, it decreased all through the experiment down to 2.34×10^4 cfu/g root. Not only bacterial density, but also culturability may change after seed inoculation: Normander et al. (1999) observed that culturable cells of *P. fluorescens* DR54-BN14 along the barley root, declined in 21 days. At the inoculation time all the cells were viable, but about 25% of them were in a viable but not culturable (VBNC) state. However, just after inoculation, the amount of VBNC cells increased to 75% and then decreased to 25% during the three following weeks.

In order to improve the efficacy and consistency of the biological control, the use of rhizosphere-competent strains is required. To fulfil this requirement, progress must be made in our knowledge of bacterial traits promoting the rhizosphere competence of fluorescent pseudomonads. Over the last two decades, many studies have been dedicated to this identification, allowing the characterization of some bacterial traits (Latour et al. 2003; Lugtenberg et al. 1991).

The spatial dynamic of the bacterial root colonization process relies on the differentiation of the root system in several morpho-physiologically distinct zones, each one being expected to release different patterns and amounts of root exudates as mirrored by the variations of microbial diversity along the root (Baudoin et al.

2001; Maloney et al. 1997; Semenov et al. 1999). Therefore, bacterial localization, organization, viability and activity of an introduced strain are expected to differ according to the root zones. Different methods and tools have been exploited in order to visualize the distribution and quantify the density of the bacteria along the root. Gradients of bacterial density have usually been recorded along the root axis. Simons et al. (1996) described variations in the number of the biocontrol *P. fluorescens* WCS365 cells along the tomato root, this number drastically (2 log orders) decreasing when getting to the root tip. Additional information on the colonization pattern of WCS365 was obtained by microscopical observations performed by scanning electron microscopy (SEM) (Chin-A-Woeng et al. 1997). After inoculation, cells appeared to replicate directly on the seed coat and develop microcolonies, mainly localized at the root base, between epidermal root cells junction. In 3-day-old plants, microcolonies were more frequent, thick and covered by a semi-transparent film. On the contrary, downward to the root tip, only single bacterial cells were found (Table 1).

P. fluorescens DF57 showed a slightly different spatial pattern of root colonization on barley seedlings (Hansen et al. 1997): on 1-day-old root, bacterial cells were mainly located on the root tip and embedded by a thick mucilage layer. As the roots became longer, bacterial cells were distributed all along the root and organized in small aggregates or strings between the root epidermal cells. However, a very low density of bacteria were detected close to the root tip. Root hairs, although well developed, were weakly colonized.

Through the combination of fluorescence, electron and confocal laser scanning (CLSM) microscopy, the cell distribution and organization of *P. fluorescens* A6RI has been characterized and related to root differentiation during the growth of tomato plants (Gamalero et al. 2004a). Just after bacterial inoculation, single cells of *P. fluorescens* A6RI were randomly distributed along the primary root, while 3 days after, bacterial cells were detected both in apex + elongation zone and hair zone. Five days after inoculation, couples of dividing bacterial cells located between epidermis cell walls were observed on the hair and collar zones. Seven days after inoculation, hardly any bacteria were found directly in the apex, while numerous bacterial cells forming strings following the longitudinal root cell walls were present in hair and collar zones. During this time course, many bacterial cells, homogeneously distributed and clustered, were detected on the root hairs. Root colonization by the strain A6RI was also characterized by dilution plating and flow cytometry, allowing the quantification of culturable and the total cells for each root zone and time (Gamalero et al. 2004a). For all sampling dates (3, 5 and 7 days) and root zones, number of total bacterial cells was always two log orders higher than that of culturable fraction, demonstrating that during time, a large fraction of bacterial cells died or became VBNC. The total cell densities did not vary significantly within each root zone while, the abundance of the culturable fraction decreased with time in the apex + elongation zone.

Gamalero et al. (2005) reported a simultaneous description of various colonization parameters of the MHB *P. fluorescens* 92 rkG5 cells along the growing root, related to morpho-physiologically different root zones of growing tomato plants. The localization, organization, and viability of 92 rkG5 cells on different root zones

Table 1 Zone of plant and presence of bacteria

		Days after inoculation				
		0	1	3	5	7
Seed		Several bacterial cells proliferating (Simons et al., 1996); mainly single bacterial cells (Tombolini et al. 1999)	Long ribbonlike bacterial cells corresponding to intracellular colonization of the seed glum; no bacterial in or below the aleuronic cell layer of the seed (Tombolini et al. 1999)			
Lower	Root tip	Few single cells (Gamalero et al. 2004; Simons et al. 1996)	Abundant bacterial cells on the mucilage layer (Hansen et al. 1997)	No bacterial cells (Gamalero et al. 2004a, 2005)	No bacterial cells (Gamalero et al. 2004a, 2005)	No bacterial cells (Gamalero et al. 2004a, 2005)
	Elongation zone	Few single cells (Simons et al. 1996)		Several single bacterial cells (Gamalero et al. 2004a, 2005) Single bacterial cells (Simons et al. 1996)	Few couple of dividing cells (Gamalero et al. 2004a, 2005)	Few couple of dividing cells (Gamalero et al. 2004a, 2005) Appearance of microcolonies (Simons et al. 1996)
Middle	Root hair zone	Few single cells (Simons et al. 1996)		Dividing cells on the younger part; microcolonies and strings on the older part (Gamalero et al. 2004a, 2005)	Dividing cells on the younger part; microcolonies and strings on the older part (Gamalero et al. 2004a, 2005)	Dividing cells on the younger part; microcolonies and strings on the older part (Gamalero et al. 2004a, 2005); strings (Hansen et al. 1997)

Upper	Collar zone	Few single cells (Simons et al. 1996)		Abundant single and couple of cells (Gamalero et al. 2004a, 2005)	Abundant single and couple of cells (Gamalero et al. 2004a, 2005)	Abundant single cells and couple of cells (Gamalero et al. 2004a, 2005)
				Strong proliferation of bacterial organized in microcolonies (Simons et al. 1996)		
Root hairs			Weak colonization (Hansen et al. 1997); no colonization (Simons et al. 1996)	Densely colonized (Gamalero et al. 2004a, 2005); no colonization (Simons et al. 1996)	Densely colonized (Gamalero et al. 2004a, 2005)	Densely colonized (Gamalero et al. 2004a, 2005); weak colonization (Hansen et al. 1997); no colonization (Simons et al. 1996)

and days were assessed by CLSM, while for each root zone and time (3, 5 and 7 days) density of culturable bacteria was assessed by dilution plating and that of VBNC and dead cells were obtained by flow cytometry. Microscopical observations showed that *P. fluorescens* 92rkG5 distribution and organization changed with space but not with time (3, 5 and 7 days). No bacterial cells were detected directly on the root tip, whereas rare single bacteria located along the root cell walls were observed in the elongation zone. On the contrary, bacterial colonization on root hair zone was intense and characterized by an increasing complexity of cell organization: evenly distributed single and dividing cells were found closer to the root tip, while microcolonies and strings were abundant closer to the collar zone. Root hairs were densely colonized by single and clustered bacterial cells, while the collar zone was colonized both by individual bacterial cells and microcolonies located along the cell walls. Quantitative analysis showed that the culturable bacterial cells, although increasing with time, were never dominant, while dead cells, prevalent along the 3-day-old root, decreased with time. The cell culturability was highest in hair zone; cells in the VBNC state occurred on the root, but they were prevalent in the apex + elongation zone of 7-day-old roots.

As the density of fluorescent pseudomonads cells decreases towards the younger part of the root, the activity of bacterial cells appeared to decrease. Using the lux-tagged *P. fluorescens* 5RL, De Weger et al. (1997) showed that the highest bacterial activity occurred on the upper part of the wheat root, while spot-like activity was recorded on the lower parts, possibly due to the presence of microcolonies. Although cells of the strains *P. fluorescens* SBW25::gfp/lux were detected on the upper part of wheat tap root, bacteria were rarely detected on lateral roots and never on root hairs (Unge and Jannson 2001).

Altogether, these data suggest the following spatio-temporal model of root colonization: after seed inoculation, most viable and active bacterial cells remain close to the inoculation site (Tombolini et al. 1999), then single cells move toward the older part through chemotactic stimulation and establish new colonies with different shapes (i.e., clusters, microcolonies, strings) mainly constituted by viable and active cells. Surprisingly, although the root tip is a major exudation compartment, it appears hardly to be colonized and also the bacterial activity remains low. This could possibly be ascribed to the intense mechanical abrasion exerted by the soil in which the root elongates (Bowen and Rovira 1976) to the presence of chemical inhibitors such as activated oxygen species or toxic compounds in root exudates (Katsuwon et al. 1993), and to the high turnover of the root cap cells. In fact, as has been demonstrated by Humphris et al. (2005), the production of border cells, acting as a disposable surface or sheath around the cap, may prevent root tip colonization by the biocontrol agent *P. fluorescens* SBW25. Besides, several components of the border cells can act as signals specifically attracting, repelling, or controlling growth and gene expression in soil microorganisms (Hawes et al. 1998).

Some fluorescent pseudomonads are not only able to colonize the root surface but were shown to colonize the root interior and the O-antigenic side chain of their outer membrane lipopolysaccharides (LPS) to differ from that of bacteria colonizing the root surface, suggesting the possible involvement of the outer membrane LPS in their ability to colonize the root interior (Van Peer et al. 1990). This was

confirmed by comparing the colonization pattern of tomato root by a *P. fluorescens* strain and its mutant affected in the O-antigenic side chain of the LPS. As shown by transmission electronic microscopy, the wild type appeared to colonizeroot cells and intracellular spaces, whereas its LPS- mutant hardly not (Duijff et al. 1997).

4 AM Fungi

The events characterizing the establishment of the arbuscular mycorrhizal symbiosis have been described in detail (Smith and Read 1997; Marsh and Schultze 2001). They have been studied either by direct observation or by means of symbiosis-defective plant mutants (Duc et al. 1989; Marsh and Schultze 2001; Paszkowski et al. 2006), a very fruitful approach.

Spore germination is followed by presymbiotic growth, which is limited (in space and time) if the hyphae do not meet any host root. After hyphal branching, the contact between the two partners is established by means of appressorium formation. Penetration into the epidermal cells has recently been described as a highly structured process, involving active participation by the host plant cell. Changes of the nucleus position, rearrangements of the cytoskeletal structures and of the endoplasmic reticulum organize a pre-penetration apparatus which defines the exact pathway of fungal penetration (Genre et al. 2005). Intraradical growth culminates in arbuscule formation, the most characteristic feature of this symbiosis, and probably the less known yet. Arbuscules are supposed to be the site of nutrient exchange, but the understanding of the cellular and molecular mechanisms of exchange is not yet complete. Arbuscules are ephemeral structures, with a life span of about 7 days and, when an arbuscule collapses, it can be substituted by a new one (Toth and Miller 1984; Alexander et al. 1988, 1989). Arbuscule turnover is associated to jasmonic acid synthesis (Hause et al. 2002) and H_2O_2 accumulation (Salzer and Boller 2000). While the fungus develops its intraradical hyphae, external mycelium is developed, finally leading to new spore formation.

Several factors can affect the establishing, developing and extent of mycorrhizal colonization. They can depend on environmental conditions (abiotic factors), on the plant species, on the previously establishment of another mycorrhizal or bacterial symbiosis. Some of the above mentioned elements will be examined.

4.1 Role of P in Mycorrhizal Colonization

A huge number of papers, often concerning investigations with diverse purposes and not necessarily aiming at the description of colonization dynamics, report data related to the fungal colonization under different levels of phosphorus availability. In general, high P availability leads to reduced or null mycorrhizal colonization (Smith and Read 1997; Vierheilig 2004a). According to the available data, plants respond to the signals from AM fungi modifying their flavonoid profiles in the

roots. The accumulation of some flavonoids leads to inhibitory effects on root colonization, like medicarpin in alfalfa, a compound that is weakly accumulated in roots with low P content (Guenoune et al. 2001). On the other hand, some flavonoids, like isovitexin 2"-O-β-glucoside in melon, can be detected only in plants with low P status and not in those with high P content (Akiyama et al. 2002). Interestingly, medicarpin content is increased in mature stages of the symbiosis, when arbuscule turnover is at work (Larose et al. 2002), and isovitexin 2'-O-β-glucoside is powerful antioxidant (Akiyama et al. 2002). It is tempting to hypothesize that arbuscule collapse is achieved by means of an oxidative burst (a well known defence mechanism), that might be triggered by the reaching of certain levels of P in the roots and inhibited by the production of antioxidants.

4.2 Autoregulation of AM Colonization

Pinior et al. (1999) reported that root exudates of plants colonized by *Glomus mosseae, G. intraradices* or *Gigaspora rosea* inhibit root colonization by *G. mosseae*. Further investigation proved that in split-root systems of barley, once AM fungi have colonized one half of the split root system, colonization of the other half by an AM fungus is significantly reduced. Colonization by *G. mosseae* in one half of the root system was reduced when pre-colonized in the other half by *G. mosseae, G. intraradices* or *Gigaspora rosea*, showing that the mechanism is not specific (Vierheilig et al. 2000). In addition, autoregulation occurs only when the first side of the split-root system is heavily colonized and not when colonization levels are low (Vierheilig 2004b). The autoregulation of colonization is not dependent on P status (Vierheilig et al. 2000), nor on carbon competition between the first fungus (early colonization of one half of the split root system) and the second one (later colonization of the second half of the split system), as discussed by Vierheiligh (2004b).

4.3 Regulation of AM Colonization by Means of Nodulation

Autoregulation of a root symbiosis was first shown for nodulation (Caetano-Anollés and Gresshoff 1991), and this system was used as a reference when describing autoregulation of mycorrhization (e.g., Vierheilig et al. 2000; Vierheilig 2004a). Furthermore, it is known that some genes involved in the root-*Rhizobium* symbiosis can also affect mycorrhizal colonization, such as enod40, their overexpression leading to faster colonization and enhanced arbuscule formation (Staehelin et al. 2001). Therefore, it was logical to guess that the two symbioses might affect each other. Catford et al. (2003) reported that nodulation (and also application of Nod factors) on one half of an alfalfa split-root system suppresses mycorrhization on the other side of the split system; similarly, a fully established mycorrhization systemically prevented both further AM colonization and nodulation. It must be

reminded that papers by Xie et al. (1995, 1998) reported that the application of Nod factors to legume roots promoted AMF colonization. However, in these two cases, the treatment with Nod factors and AMF inoculation were performed simultaneously and plants were not grown with a split-root system. Therefore, it seems possible that Nod factors promote mycorrhization locally, and suppress it systemically by means of some autoregolatory mechanism.

4.4 Exudates

Plant roots and AM fungi perceive each other before penetration or appressorium formation (Salzer and Boller 2000; Vierheilig and Piché 2002). In fact, non-susceptible plants (such as Brassicaceae or plants with high P status) show altered levels of some compounds [like the defence-related hydrolases β-1,3-glucanase and chitinase in some Brassicaceae (Vierheilig et al. 1994), or like formononentin in alfalfa plants with high P status (Volpin et al. 1994)]. On the fungus side, root exudates of tomato mutants, resistant to mycorrhization, can suppress spore germination and hyphal growth (David-Schwartz et al. 2003; Gadkar et al. 2003), and some root exudates of non-mycorrhizal plants are required in order to observe hyphal elongation and branching (Graham 1982; Elias and Safir 1987; Bécard and Piché 1989; Tawaraya et al. 1996; Nagahashi and Douds 2000; Buée et al. 2000). Exudates extracted from mycorrhizal roots showed inhibitory effects on root colonization (Pinior et al. 1999). A molecule promoting hyphal branching has recently been isolated and purified by Akyiama et al. (2005), confirming that factors of plant origin are essential for the steps of the mycorrhizal symbiosis that precede the plant-fungus physical contact. Susceptible host plants produce sesquiterpenes that induce branching of the hyphae prior to direct contact with the roots. Strigolactones are germinating stimulants for some parasitic weeds, belonging to the genera *Striga* and *Orobanche* (Bouwmeester et al. 2003). The role of a sesquiterpene lactone (namely 5-deoxystrigol, strigolactone) as branching factor for mycorrhizal fungi has been firstly demonstrated by isolating this molecule from root exudates of *Lotus japonicus*, and by showing that other natural (sorgolactone and strigol) and synthetic (GR24) molecules induce similar effects on germinating spores of *G. margarita* (Akiyama et al. 2005). In agreement with the report by Tamasloukht et al (2003), showing that *G. rosea* respond to branching factors increasing its respiratory activity and mitochondrion number before starting to branch, the strigolactone analogue GR24 increased the number of mitochondria, their motility and modified their shape and distribution in the hyphae of *G. rosea*. The synthetic strigolactone analogues GR24 and GR7 were active with similar effects on phylogenetically distant fungi, like *G. margarita*, and two *Glomus* species, *G. intraradices* (also including increased respiration rates for this fungus) and *G. claroideum* (Besserer et al. 2006).

Experiments with split-root systems suggest that the observed alterations in the exudation patterns following mycorrhization seem not to be limited to mycorrhizal

roots, but to also occur in the non-mycorrhizal portion of AM plants, clearly indicating that they are systemic (Vierheilig et al. 2003), similarly to some defence responses observed in AM plants challenged with pathogenic fungi (Cordier et al. 1998).

4.5 Interactions Between Fluorescent Pseudomonads and AM Fungi

Considering colonization patterns of both types of microorganisms – AMF and fluorescent pseudomonads – several important issues will be briefly addressed below. More detailed information is available in recent reviews related to the interactions between AMF and bacteria at the root level (Bianciotto and Bonfante 2002; Johansson et al. 2004).

Reciprocal interactions between AM and fluorescent pseudomonads are expected to occur during the presymbiotic growth, and during the infection and colonization processes of roots by AMF. Indeed, fluorescent pseudomonads have been proposed as potential BCA, and their modes of action are expressed during the two phases of the life cycle of pathogenic fungi. During the fungal saprophytic phase, fluorescent pseudomonads may suppress fungal growth through microbial antagonism resulting from antibiosis and/or siderophore-mediated iron competition (Haas and Défago 2005). During the fungal parasitic phase, fluorescent pseudomonads may suppress their colonization through the elicitation of plant defence reactions (Van Loon et al. 1998). Therefore, possible impact of these bacterial actions on the AMF needs to be evaluated to assess possible side effects of BCA inoculation. However, several reports have suggested or indicated that microbial antagonists of fungal pathogens do not exert an antimicrobial effect against AMF (Barea et al. 1998; Edwards et al. 1998; Vasquez et al. 2000; Barea et al. 2002). Nevertheless, Paulitz and Linderman (1989) have reported a delay of the germination of *G. etunicatum* in raw soil by the antibiotic producer pseudomonad strains 3871 and 2–79. We have also recently shown by transmission electronic microscopy that specific strains of AMF and BCA fluorescent pseudomonad can live together within the same root cell (Gianinazzi-Pearson, Arnould, Loison, and Lemanceau, unpublished data), suggesting that this bacterial BCA was not suppressing the endophyte root colonization of the inoculated AMF.

Bacteria, including fluorescent pseudomonads, have been described to even promote mycorrhization, these bacteria being called mycorrhiza helper bacteria (MHB) (Garbaye 1994). This concept was nicely illustrated with a *P. fluorescens* promoting the symbiosis between Douglas fir and the ectomycorrhizal fungus *Laccaria bicolor* (Duponnois and Garbaye 1991); reciprocally the density of that bacterial strain was significantly higher when inoculated in mycorrhized than in nonmycorrhized Douglas fir (Frey-Klett et al. 1997). This concept was also illustrated by the mutual enhancement of root colonization in *Glomus* and *Pseudomonas* dual inoculation (Meyer and Linderman 1986a). Other examples of AM promotion by fluorescent pseudomonads have reported in the literature (Bhowmik and Singh 2004; Ravnskov

and Jakobsen 1999; Gamalero et al. 2004b; Zaidi and Khan 2005). A *Pseudomonas* strain was even shown to promote both ectomycorrhization and endomycorrhization (by *G. intraradices*) of *Acacia holosericea* (Duponnois and Planchette 2003). There is high diversity among fluorescent pseudomonads in rhizospheres (Lemanceau et al. 1995) and some of them were shown not to impact AM either positively or negatively (Edwards et al. 1998; Paulitz and Linderman 1989). Similarly, densities of inoculated pseudomonads appear to be either promoted, unaffected (Edwards et al. 1998) or even depressed (Berta et al., unpublished data; Paulitz and Linderman 1989) when co-inoculated with AMF. Some pseudomonads have the ability to attach to spores and hyphae of AMF as shown with *G. margarita in vitro* (Bianciotto et al. 1996); and Mayo et al. (1986) have shown that, among bacteria attached to spores of *G. versiforme*, some strains of *Pseudomonas* were shown to promote *in vitro* spore germination and germ-tube hyphal growth and branching. AMF, known to modify root architecture and physiology (see the following section), are expected to influence the quantity and quality of root exudates impacting consequently the diversity and activity of the bacterioflora. Modifications of the root architecture and physiology induced are expected to influence the quantity and quality of root exudates impacting consequently the diversity and activity of the bacterioflora. These influences within the so-called mycorrhizosphere (Linderman 1988) have been shown (Ames et al. 1984; Andrade et al. 1997; Meyer and Linderman 1986b; Vasquez et al. 2000) as was also the case with ectomycorrhizae (Frey-Klett et al. 1997, 2005). Sood (2003) reported that tomato plants inoculated with *G. fasciculatum* exert a stronger chemotactic effect toward an introduced *P. fluorescens* than the noninoculated control. Recently, Rillig et al. (2006) have even further indicated that phylogeny of AMF predicts community composition of symbiosis-associated bacteria. Mougel et al. (2006) have clearly demonstrated shifts in the genetic structure of bacteria communities induced by AM when comparing bacterioflora associated with mycorrhized (wild type) and non-mycorrhized (Myc- mutant) roots of *Medicago truncatula*. Populations preferentially associated with mycorrhized roots are expected at least not to suppress AM or better to promote them. This could then be a strategy to isolate bacterial strains adapted to the mycorrhizosphere and promoting mycorrhization. This hypothesis is currently being evaluated through a cooperative work between our two groups (Pivato et al., unpublished data).

Further research is required to identify possible mechanisms mediating the reciprocal promotion of colonization by strains of AMF and fluorescent pseudomonads. Some attempts were already made to identify which fraction – according to their molecular weight – was involved in the growth promotion of *Glomus fistulosum* by a *P. putida* (Vosatka and Gryndler 1999). Possible strategies would rely on (1) genomic analysis of fungal-bacterial interactions as proposed by Frey-Klett and Garbaye (2005), and on (2) comparison the metaproteome of the AMF and pseudomonad – following that proposed by Maron et al. (2008) – when inoculated separately and in combination. Patterns of root colonization of AMF and pseudomonad when inoculated separately and in combination also need to be better characterized along the roots by microscopic observations. All these studies need to take in

account the spatio-temporal heterogeneity since the effect of one organism on the other may differ with time (Meyer and Linderman 1986a; Paulitz and Linderman 1989) and probably with the root zones expressing different root exudation patterns. Possible specificity of these interactions of these interactions also needs to be considered as indicated by Paulitz and Linderman (1989) and Vasquez et al. (2000).

4.6 Root Colonization by Fluorescent Pseudomonads and AM Fungi: Consequences for Plant Health

Root system morphology is genetically determined, but the number, placement, and direction of growth of each root in the system is highly variable, even among genetically identical plants (Malamy 2005). Environmental factors (nutrient and water availability, temperature, gravity, wind, soil density and texture, presence of xenobiotics, etc.) (Malamy 2005; Chiatante et al. 2005), and interactions with soil microorganisms, as rhizobacteria, pathogenic and endophytic fungi, including mycorrhizal fungi (Berta et al. 2002, 2005; Gamalero et al. 2004b; Mucciarelli et al. 2002) are among the main cause of the developmental plasticity of roots. The establishment of arbuscular mycorrhizal symbiosis does not influence root and root apex morphology as do ectomycorrhizae. For this reason, until the middle of the 1980s, the notion that arbuscular mycorrhizal fungi had no influence on either root morphogenesis or the dynamics of host root meristems, was virtually an article of faith, as only differences between weights of mycorrhizal and nonmycorrhizal plants were occasionally found (Harley and Smith 1983). Only in 1990 was the first paper about the morphogenetic modifications induced by an AM fungus published (Berta et al. 1990). Up to date, many authors have shown that some plant species respond to the presence of the AM fungi with modalities that vary from one host to another, and from one fungus to another. Plant colonization by AM fungi is generally associated with enhanced growth of the whole plant and of the root system, the latter mainly by formation of new lateral roots which give rise to a more branched root system. However, the order of the roots involved varies. In the monocot *Allium porrum* colonized by *Glomus* sp., the number of lateral roots per unit length of adventitious root is significantly higher in AM plants (Berta et al. 1990). On the contrary, the degree of branching of the main axes of the woody dicotyledonous plants *Prunus cerasifera* (Berta et al. 1995) and *Platanus acerifolia* (Tisserant et al. 1992, 1996) is almost unaltered, and the greatest morphological effect of AM colonization is the intensity of branching of first order lateral roots in the former and of the development of laterals of higher order in the latter. AM root systems are hence characterized by modifications in the hierarchical appearance of different root orders, which cause a different contribution of the different root orders to the whole root apparatus (Scannerini et al. 2002). Recent findings on *M. truncatula* show that AM fungi increase the number of lateral roots at a very early stage of the

root–fungus interaction, before hyphae have established contact with roots, suggesting that secretion of diffusible factors could be involved (Oláh et al. 2005).

Even if AM fungi never penetrate the root apical meristems, it has been demonstrated that AM fungi can alter their size and activity; data on this matter, however, are very scarce and the only results were obtained on *A. porrum* plants infected by *Glomus* sp. E3 (Berta et al. 1990; 1991). In *A. porrum*, the apices of adventitious roots of mycorrhizal plants do not change in structure with respect to those of uninfected ones but increase drastically in size and have a larger meristem and quiescent center (Fusconi et al. 1994). In spite of the larger size, AM apices show a lower metabolic activity than controls (Fusconi et al. 1994), the mitotic cycles of AM apical meristems become longer with increasing infection (Berta et al. 1991), and chromosomal aberrations, consisting of C-mitosis, occur. Lastly, AM fungi block meristem activity and mycorrhizal plants possess a higher percentage of inactive apices, i.e., of apices that become "parenchymatous" or "determinate" according to Berta et al. (1993) and Varney and McCully (1991), respectively; meristem cells lose their mitotic activity and undergo a limited cell distension and vacuolization. The increase of apex size and the lowering of the mitotic activity are the direct cause of the shortening and thickening of adventitious roots observed in mycorrhizal plants (Berta et al. 2002). Root growth inhibition observed in *A. porrum* is not a general characteristic of mycorrhizal roots, for example, it does not occur in *M. truncatula* (Oláh et al. 2005) and, hence, it may be related to a specific strategy of growth (or of resource acquisition). One of the main benefits of mycorrhizal fungi on plants is the increased uptake of nutrients, mainly of phosphate, from the soil and, as a consequence, the P status of the plant tissues is higher in mycorrhizal than in nonmycorrhizal plants grown on the same medium (Fusconi et al. 2005). The tissue P content may be, by itself, a cause of growth alteration. In *A. porrum*, about 100 μM P causes an increase of root branching and a lengthening of the mitotic cycle comparable to those induced by mycorrhization at low P level (about 3 μM). However, the slowing down of the mitotic cycle does not cause a block of the mitotic activity, as do mycorrhizas, and result in a slow and steady growth of the adventitious roots (Fusconi et al. 2000).

Hormones control most of the characteristics of the root system, including primary root growth and formation of lateral roots (López-Bucio et al. 2003), and morphogenetic effects of AM fungi on the root system, dependent or not by P, are probably mediated by changes in hormone synthesis, transport or sensitivity (Beyrle 1995). However, their precise role in the interaction is still unknown. Evidence for phytohormone involvement in AM comes mainly from application experiments or from increases in their endogenous levels observed during mycorrhization (Isayenkov et al. 2005). Altered levels of auxin (Kaldorf and Ludwig-Müller 2000; Torelli et al. 2000) have been reported in mycorrhizal plants. However, in *M. truncatula*, the hypothetical diffusible AM fungal factors is unlikely to be an auxin-like substance because of the different morphogenetic effect following auxin administration, whilst Nod factors stimulate lateral root formation in a way similar to that of AM fungi, suggesting a general role of these molecular signals as plant growth regulators, in addition to their role during the nodulation

process (Oláh et al. 2005). A rise in endogenous levels of cytokinin in AM plants was also observed (Barker and Tagu 2000; Torelli et al. 2000; Fitze et al. 2005), and in *Hordeum vulgare* and *M. truncatula* an increase of jasmonate has been shown (Hause et al. 2002; Stumpe et al. 2005) that could play an indirect role in mycorrhization via the action of cytokinins (Isayenkov et al. 2005). Besides, other more specific aspects of the mycorrhizal interaction may be influenced by hormones: the lengthening of the mitotic cycle reported for mycorrhizal root apices of *A. porrum* could tentatively be related to an increase of ABA, as it has an inhibitory effect on the progression of the cell cycle, and it was found at high levels in AM *Zea mays* roots (Danneberg et al. 1992), and in the extraradical hyphae of *Glomus* sp. isolates (Esch et al. 1994). In addition, other hormonal substances such as ethylene (Vierheilig et al. 1994) and gibberellins (Shaul-Keinan et al. 2002) may be involved in AM morphogenesis, depending on different plant and fungus species. Among the morphogenetic, nonhormonal factors involved in AM root morphogenesis, we cannot exclude a role of the hyperpolarization of root cortical cells, shown in *A. porrum* + *Glomus* E3 mycorrhizae (Fieschi et al. 1992).

The precocious inactivation and loss of the meristem of the AM root apices of *A. porrum* does not lead to the senescence of the differentiated, colonized root tissues. On the contrary, in these tissues senescence is delayed (Lingua et al. 1999). An opposite situation occurs in the roots of trees (e.g., *Platanus acerifolia, Populus* sp.) where AM fungi promote the senescence of the fine rootlets and, consequently, their turnover, so giving a significant contribution to biogeochemical cycling (Atkinson et al. 1994). The different fungal effect on root longevity could be related to root function, as adventitious roots are primarily engaged in exploration, whilst higher order roots of trees are primarily engaged in absorption. These two strategies (delayed senescence and root turnover) enable AM fungi to accomplish the same result, namely a more vital and efficient root system.

Plant growth and root system structure, as already said, are also influenced by soil microrganisms other than mycorrhizal fungi. The ability of fluorescent pseudomonads to promote plant growth has been reported many times since the 1980s (Burr et al. 1978; Bakker et al. 1987; Glick 1995; Gamalero et al. 2004b). The mechanisms involved are usually classified in (1) a direct stimulation through improved mineral nutrition and phytohormone production, and (2) an indirect stimulation via the suppression of soilborne diseases and enhancement of plant tolerance to biotic and abiotic stresses (Whipps 2001). Plant growth promotion induced by bacteria has been mostly expressed as increased plant weight and root length. Recently, Gamalero et al. (2002, 2004b) have shown that fluorescent pseudomonads may also affect root morphogenesis and architecture. More specifically the tested strains induced an increased total root length, surface area and volume (Gamalero et al. 2002, 2004b), which may be related to their ability to produce hormones and to improve plant mineral nutrition (Gamalero et al. 2004b). In turn, root architecture influences bacterial colonization, as different root zones are expected to produce different exudates (Jaeger et al. 1999; Baudoin et al. 2001), and evidence exists indicating that root exudates can take part in the signaling events that initiate the execution of the interactions between plants and microorganisms (Bais et al. 2006).

AM fungi can reduce root diseases caused by soil-borne pathogens (Harrier and Watson 2004), and alteration of root architecture and longevity is an important factor contributing to the induced resistance. Synergistic action with rhizobacteria also plays an important role: in tomato plants infected by the soil-borne pathogen *Rhizoctonia solani*, it has recently been shown that efficient disease suppression by *G. mosseae* and by pseudomonads is associated with combined effects both on the pathogen and on the plant (Berta et al. 2005). In addition, some events occur as competition for colonization and infection sites and for photosyntates, including localized morphological and chemical changes in root tissues (cell wall lignification, callose production), modification of the rhizodeposition altering the chemotaxis to the root in the pathogens, and microbial changes in the mycorrhizosphere (see reviews of Azcón-Aguilar and Barea 1996; Linderman 2000; Harrier and Watson 2004). Surprisingly, antifungal metabolites released by bacterial BCA in the rhizosphere do not seem to affect the growth and symbiosis establishment of AM fungi (Barea et al. 1998).

Anyway, the signals involved in the induction of the resistance and the possible involvement of plant hormones are largely unknown (Hause and Fester 2005). As mentioned above, AM can induce high levels of jasmonic acid (JA) in a number of species (Hause et al. 2002; Stumpe et al. 2005) and evidence from genetic analyses of plant mutants and transgenics, that are affected in the biosynthesis or perception of JA, indicate a role of JA in basal resistance as well as in resistance against pathogens with a (hemi-) biotrophic lifestyle, possibly by acting in concert with other defence signaling pathways (Pozo et al. 2005). A recently hypothesized possibility is that apocarotenoids occurring in many mycorrhizal roots (as cyclohexenone and mycorradicin derivatives) could be involved in the production of signaling molecules and in the control of fungal colonization or protection against soil-borne pathogens (Strack and Fester 2006).

In conclusion, all the information presented here supports the idea of root systems as very plastic structures, influenced by the environment and by the soil microorganisms, but also able to influence them: plants control colonization degree (Vierheilig 2004a, 2004b), mycelium growth and interconnectedness (Giovanetti et al. 2004) and, depending on their root system structure and physiology, are differently independent from mycorrhization (Fitter 2004; Azcón and Ocampo 1981) and differently colonized by rhizobacteria

5 Conclusions

Plants are autotrophic and release a significant part of their photosynthates in the rhizosphere. These organic compounds support the multiplication and activity of a high density of soilborne microorganisms which are mostly heterotrophic, among which are the fluorescent pseudomonads. Some of these populations are favorable to the growth and health of the plant supporting them. Similarly, plant roots provide to AMF organic compounds required for their metabolism and, reciprocally, they promote plant nutrition,

growth and health. Mutualistic microflora either symbiotic (AMF) or not (fluorescent pseudomonads) associated with the roots contribute clearly to the adaptation of the host-plant to low fertility environment and also protect them against various biotic and abiotic stresses (Smith and Read 1997; Barea et al. 2002; Pozo et al. 2002; Berta et al. 2005). Several authors have proposed that AM have contributed to the colonization of early land plants (Selosse and Le Tacon 1998; Redecker et al. 2000).

The AM association represents an ancient symbiosis with fossil evidence dating back 400 million years (Remy et al. 1994). The long, joint evolution of plants and AM fungi is suspected to have not occurred independently of the resident microflora. Indeed, Offre et al. (2007) have recently shown that the genetic structure of bacterial communities associated with mycorrhized and nonmycorrhized roots of *M. truncatula* differs significantly, and that bacteria belonging to Burkhoderiales are preferentially associated with mycorrhized roots. The impact of AM on associated bacterioflora including fluorescent pseudomonads could be ascribed to variations of plant physiology, root architecture and consequently root exudation induced by the fungal symbiosis. Reciprocally, bacterial populations favored by the AM are suspected to at least not depress but even better promote mycorhization. This hypothesis is currently being evaluated (Pivato et al., unpublished data).

To interact together in the rhizosphere, AMF and bacteria must either be located at the same sites or induce systemic modifications of their host-plant. Further research is therefore required to localize both types of organisms at the root level, in order to discriminate what are the contributions of microbial interactions and of plant-mediated interactions on the reciprocal effects of each type of organisms. Analysis of fungal and bacterial traits involved in the possible reciprocal beneficial effects on each organism on the other also need to be further explored by functional genomic approaches. On top of this basic research, further experiments are also needed to identify strains of AMF and fluorescent pseudomonads, compatible together or even promoting each other, that would combine different beneficial effects on the plants as already described by Berta et al. (2005).

References

Akiyama K, Matsuoka H, Hayashi H (2002) Isolation and identification of a phosphate deficiency-induced C-glycosylflavonoid that stimulates arbuscular mycorrhiza formation in melon roots. Mol Plant Microbe Interact 15:334–340

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Alexander T, Meier R, Toth R, Weber HC (1988) Dynamics of arbuscule development and degeneration in mycorrhizas of *Triticum aestivum* L. and *Avena sativa* L. with reference to *Zea mays* L. New Phytol 110:363–370

Alexander T, Meier R, Toth R, Weber HC (1989) Dynamics of arbuscule development and degeneration in onion bean and tomato, with reference to vesicular-arbuscular mycorrhizas with grasses. Can J Bot 67:2505–2513

Ames S, Reid CPPP, Ingham ER (1984) Rhizosphere bacterial population responses to root colonization by a vesicular-arbuscular mycorrhizal fungus. New Phytol 96:555–563

Andrade G, Milhara KL, Linderman RG, Bethlenfalay GJ (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular-mycorrhizal fungi. Plant Soil 192:71–79

Atkinson D, Berta G, Hooker JE (1994) Impact of root colonization in root architecture, root longevity and the formation of growth regulators. In: Gianinazzi S, Schüepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystem. Birkhäuser, Basel, pp 89–99

Azcón-Aguilar C, Barea JM (1996) Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - an overview of the mechanisms involved. Mycorrhiza 6:457–464.

Azcón R, Ocampo JA (1981) Factors affecting the vesicular-arbuscular infection and mycorrhizal dependency of thirteen wheat cultivar. New Phytol 87:677–685

Bago B, Vierheilig H, Piché Y, Azcon-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Bakker PAHM, Bakker AW, Marugg JD, Weisbeek PJ, Schippers B (1987) Bioassay for studying the role of siderophores in plant growth stimulation by fluorescent *Pseudomonas* spp. in short potato rotations. Soil Biol Biochem 19:443–444

Bansal M, Mukerji KG (1994) Positive correlation between VAM-induced changes in root exudation and mycorrhizosphere mycoflora. Mycorrhiza 5:39–44

Barea JM, Andrade G, Bianciotto V, Dowling D, Lohrke S, Bonfante P, O'Fara F, Azcon-Aguilar C (1998) Impact on arbuscular mycorrhiza formation of *Pseudomonas* strains used as inoculants for the biocontrol of soil-borne plant fungal pathogenes. Appl Environ Microbiol 64:2304–2307

Barea JM, Gryndle M, Lemanceau P, Schüepp H, Azcon R (2002) The rhizosphere of mycorrhizal plants, 1–18. In: Gianinazzi S, Schuepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkäuser, Basel

Barker S, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbioses. J Plant Growth Regul 19:144–154

Baudoin E, Benizri E, Guckert A (2001) Metabolic fingerprinting of microbial communities from distinct maize rhizosphere compartments. Eur J Soil Biol 37:85–93

Bécard G, Piché Y (1989) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis. Appl Environ Microbiol 55:2320–2325

Berta G, Fusconi A, Trotta A, Scannerini S (1990) Morphogenetic modifications induced by the mycorrhizal fungus *Glomus* strain E3 in the root system of *Allium porrum* L. New Phytol 114:207–215

Berta G, Tagliasacchi AM, Fusconi A, Gerlero D, Trotta A, Scannerini S (1991) The mitotic cycle in root apical meristems of *Allium porrum* L. is controlled by the endomycorrhizal fungus *Glomus* sp. strain E3. Protoplasma 161:12–16

Berta G, Fusconi A, Trotta A (1993) VA mycorrhizal infection and the morphology and function of root systems. Env Exp Bot 33:159–173

Berta G, Trotta A, Fusconi A, Hooker JE, Munro M, Atkinson D, Giovannetti M, Morini S, Fortuna P, Tisserant B, Gianinazzi-Pearson V, Gianinazzi S (1995) Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera*. Tree Physiol 15:281–293

Berta G, Fusconi A, Hooker JE (2002) Arbuscular mycorrhizal modifications to plant root systems. In: Gianinazzi S, Schuepp H (eds) Mycorrhizal technology: from genes to bioproducts – achievement and hurdles in arbuscular mycorrhizal research. Birkhauser, Basel, pp 71–101

Berta G, Sampò S, Gamalero E, Massa N, Lemanceau P (2005) Suppression of *Rhizoctonia* root-rot of tomato by *Glomus mosseae* BEG12 and *Pseudomonas fluorescens* A6RI is associated with their effect on the pathogen growth and on root morphogenesis. Eur J Plant Pathol 111:279–288

Besserer A, Puech-Pagès V, Kiefer P, Gomez-Roldan V, Jauneau A, Roy S, Portais J-C, Roux C, Bécard G, Séjalon-Dumas N (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLOS Biol 4:1239–1247

Beyerle H (1995) The role of phytohormones in the function and biology of mycorrhizas In: Varma A, Hock B (eds) Mycorrhiza: structure, molecular biology and function. Springer, Heidelberg, pp 365–390

Bhowmik SN, Singh CS (2004) Mass multiplication of AM inoculum: effect of plant growth-promoting rhizobacteria and yeast in rapid culturing of *Glomus mosseae*. Curr Sci India 86:705–709

Bianciotto V, Bonfante P (2002) Arbuscular mycorrhizal fungi: a specialised niche for rhizospheric and endocelluar bacteria. Antonie Van Leeuwenhoek 81:365–371

Bianciotto V, Minerdi D, Perotto S, Bonfante P (1996) Cellular interactions between arbuscular fungi and rhizosphere bacteria. Protoplasma 193:123–131

Bouwmeester HJ, Matusova R, Zhongkui S, Beale MH (2003) Secondary metabolite signalling in host-parasitic plant interactions. Curr Opin Plant Biol 6:358–364

Bowen GD, Rovira AD. 1976. Microbial colonization of plant roots. Annu Rev Phytopathol 14:121–144

Buée M, Rossignol M, Jauneau A, Ranjeva R, Bécard G (2000) The pre-symbiotic growth of arbuscular mycorrhizal fungi is induced by a branching factor partially purified from plant root exudates. Mol Plant Microbe Interact 13:693–698

Bull CT, Weller DM, Thomashow LS (1991) Relationship between root colonisation and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2–79. Phytopathology 81:954–959

Burr TJ, Schroth MN, Suslow T (1978) Increased potato yields by treatment of seed pieces with specific strain of *Pseudomonas fluorescens* and *P. putida*. Phytopathology 68:1377–1383

Caetano-Anollés G, Gresshoff PM (1991) Alfalfa controls nodulation during the onset of *Rhizobium*-induced cortical cell division. Plant Physiol 95:366–373

Catford JG, Staehelin C, Lerat S, Piché Y, Vierheilig H (2003) Suppression of arbuscular mycorrhizal colonization and nodulation in split-root systems of alfalfa after pre-inoculation and treatment with Nod factors. J Exp Bot 54:1481–1487

Chiatante D, Di Iorio A, Scippa GS (2005) Root responses of *Quercus ilex* seedlings to drought and fire. Plant Biosyst 139:198–208

Chin-A-Woeng TFC, de Priester W, van der Bij A, Lugtenberg BJJ (1997) Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. Mol Plant Microbe Interact 10:79–86

Cordier C, Pozo MJ, Barea JM, Gianinazzi S, Gianinazzi-Pearson V (1998) Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. Mol Plant Microbe Interact 11:1017–1028

Danneberg G, Latus C, Zimmer W, Hundeshagen B, Schneider-Poetsc H Hj, Bothe H (1992) Influence of vesicular-arbuscular mycorrhiza on phytohormone balance in maize (*Zea mays* L.). J Plant Physiol 141:33–39

David-Schwartz R, Gadkar V, Wininger S, Bendov R, Galili G, Levy AA, Kapulnik Y (2003) Isolation of a premycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization. Mol Plant Microbe Interact 16:382–388

De Weger LA, Kuiper I, van der Bij AJ, Lugtenberg BJJ (1997) Use of lux based procedure to rapidly visualize root colonization by *Pseudomonas fluorescens* in the wheat rhizosphere. Antonie Van Leeuwenhoek 72:365–372

Duc G, Trouvelot A, Gianinazzi-Pearson V, Gianinazzi S (1989) First report on non-mycorrhizal plant mutants (Myc-) obtained in pea (*Pisum sativum* L.) and fababean (*Vicia faba* L.). Plant Sci 60:215–222

Duijff BJ, Gianinazzi-Pearson V, Lemanceau P (1997) Involvement of the outer membrane lipolysaccharides in the endophytic colonization of tomato roots by biocontrol *Pseudomonas fluorescens* strain WCS417r. New Phytol 135:325–334

Duponnois R, Garbaye J (1991) Effect of dual inoculation of Douglas fir with the ectomyccorhizal fungus *Laccaria laccata* and mycorrhization helper bacteria (MHB) in two bare-root forest nurseries. Plant Soil 138:169–176

Duponnois R, Planchette C (2003) A mycorrhiza helper bacterium enhances ectomycorrhizal and endomychorizal symbiosis of Australian *Acacia* species. Mycorrhiza 13:85–91

Edwards SG, Young JPW, Fitter AH (1998) Interactions between *Pseudomonas fluorescens* biocontrol agents and *Glomus mosseae*, an arbuscular mycorrhizal fungus, within the rhizosphere. FEMS Microbiol Lett 116:297–303

Elias KS, Safir GR (1987) Hyphal elongation of *Glomus fasciculatus* in response to root exudates. Appl Environ Microbiol 53:1928–1933

Esch H., Hundeshagen B., Schneider-Poetsch H, Bothe, H (1994) Demonstration of abscisic acid in spores and hyphae of the arbuscular-mycorrhizal fungus *Glomus* and the N_2-fixing cyanobacterium *Anabaena variabilis*. Plant Sci 99:9–16

Fieschi M, Alloatti G, Sacco S, Berta G (1992) Membrane potential hyperpolarisation in vesicular arbuscular mycorrhizae of *Allium porrum* L.: a non-nutritional long-distance effect of the fungus. Protoplasma 168:136–140

Fitter AH (2004) Magnolioid roots - hairs, architecture and mycorrhizal dependency. New Phytol 164:15–16.

Fitze D, Wiepning A, Kaldorf M, Ludwig-Müller J (2005) Auxins in the development of an arbuscular mycorrhizal symbiosis in maize. J Plant Physiol 162:1210–1219

Frey-Klett P, Garbaye J (2005) Mycorrhiza helper bacteria: a promising model fort he genomic analysis of fungal-bacterial interactions. New Phytol 168:4–8

Frey-Klett P, Pierrat JC, Garbaye J (1997) Location and survival of mycorrhiza helper *Pseudomonas fluorescens* during establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir. Appl Environ Microbiol 63:139–144

Frey-Klett P, Chavatte M, Clausse M-L, Courrier S, Le Roux C, Raaijmakers J, Martinotti MG, Pierrat J-C, Garbaye J (2005) Ectomyccorhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. New Phytol 165:317–328

Fujishige NA, Kapadia NN, Hirsh AM (2006) A feeling for the micro-organism: structure on a small scale. Biofilms on plant roots. Bot J Linn Soc 150:79–88

Fusconi A, Tagliasacchi AM, Scannerini S, Trotta A, Gnavi E, De Padova S (1994) Root apical meristems of arbuscular mycorrhizae of *Allium porrum* L. Env Exp Bot 43:181–193

Fusconi A, Tagliasacchi AM, Berta G, Trotta A, Brazzaventre S, Ruberti F, Scannerini S (2000) Root apical meristems of *Allium porrum* L. as affected by arbuscular mycorrhizae and phosphorus. Protoplasma 214:219–226

Fusconi A, Lingua G, Trotta A, Berta G (2005) Effects of arbuscular mycorrhizal colonization and phosphorus application on nuclear ploidy in *Allium porrum* plants. Mycorrhiza 15:313–321

Gadkar V, David-Schwartz R, Nagahashi G, Douds DD, Wininger S, Kapulnik Y (2003) Root exudates of pmi tomato mutant M161 reduces AM fungal proliferation *in vitro*. FEMS Microbiol Lett 223:193–198

Gamalero E, Martinotti MG, Trotta A, Lemanceau P, Berta G (2002) Morphogenetic modifications induced by *Pseudomonas fluorescens* A6RI and *Glomus mosseae* BEG12 in the root system of tomato differ according to plant growth conditions. New Phytol 155:293–300

Gamalero E, Lingua G, Caprì FG, Fusconi A, Berta G, Lemanceau P (2004a) Colonization pattern of primary tomato roots by *Pseudomonas fluorescens* A6RI characterized by dilution plating, flow cytometry, fluorescence, confocal and scanning electron microscopy. FEMS Microbiol Ecol 48:79–87

Gamalero E, Trotta A, Massa N, Copetta A, Martinotti MG, Berta G (2004b) Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14:185–192

Gamalero E, Lingua G, Tombolini R, Avidano L, Pivato B, Berta G (2005) Colonization of tomato root seedling by *Pseudomonas fluorescens* 92 rkG5: spatio-temporal dynamics, localization, organization, viability and culturability. Microb Ecol 50:289–297

Garbaye J (1994) Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytol 128:197–210

Genre A, Chabaud M, Timmers T, Bonfante P, Barker DG (2005) Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. Plant Cell 17:3489–3499

Giovannetti M, Hepper CM (1985) Vesicular-arbuscular mycorrhizal infection in *Hedysarum coronarium* and *Onobrychis viciifolia*: host-endophyte specificity. Soil Biol Biochem 17:899–900

Giovannetti M, Sbrana C, Avio L (2004) Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. New Phytol 164:175–181

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Graham JH (1982) Effect of citrus root exudates on germination of chlamydospores of the vesicular-arbuscular mycorrhizal fungus, *Glomus epigaeum*. Mycologia 74:831–835

Guenoune D, Galili S, Phillips DA, Volpin H, Chet I, Okon Y, Kapulnik Y (2001) The defense response elicited by the pathogen *Rhizoctonia solani* is suppressed by colonization of the AM-fungus *Glomus intraradices*. Plant Sci 160:925–932

Haas D, Défago G (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat Rev Microbiol 3:307–319

Hansen M, Kragelund L, Nybroe O, Sørensen J (1997) Early colonization of barley roots by *Pseudomonas fluorescens* studied by immunofluorescence technique and confocal laser scanning microscopy. FEMS Microbiol Ecol 23:353–360

Harley JL, Smith SE (1983) Mycorrhizal symbiosis, Academic Press, London

Harrier LA, Watson CA (2004) The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. Pest Manage Sci 60:149–157

Hause B, Fester T (2005) Molecular and cell biology of arbuscular mycorrhizal symbiosis. Planta 221:184–196

Hause B, Maier W, Miersch O, Kramell R, Strack D (2002) Induction of jasmonate biosynthesis in arbuscular mycorrhizal barley roots. Plant Physiol 130:1213–1220

Hawes MC, Brigham LA, Wen F, Woo HH, Zhu Y (1998) Function of root border cells in plant health: pioneers in the rhizosphere. Ann Rev Phytopathol 36:311–327

Humphris SN, Bengough AG, Griffiths BS, Kilham K, Rodger S, Stubbs V, Valentine TA, Young IM (2005) Root cap influences root colonization by *Pseudomonas fluorescens* SBW25 on maize. FEMS Microbiol Ecol 54:123–130

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B (2005) Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. Plant Physiol 139:1401–1410

Jaeger III CH, Lindow SE, Miller W, Clark E, Firestone MK (1999) Mapping of sugar and amino acid availability in soil around roots with bacterial sensors of sucrose and tryptophan. Appl Environ Microbiol 65:2685–2690

Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS Microbiol Ecol 48:1–13

Kaldorf M, Ludwig-Müller J (2000) AM fungi affect the root morphology of maize by increasing indole-3-butyric acid biosynthesis. Physiol Plant 109:58–67

Katsuwon J, Zdor R, Anderson AJ (1993) Superoxide dismutase activity in root-colonizing pseudomonads. Can J Microbiol 39:420–429

Larose G, Chenevert R, Moutoglis P, Gagne S, Piché Y, Vierheilig H (2002) Flavonoid levels in roots of *Medicago sativa* are modulated by the developmental stage of the symbiosis and the root colonizing arbuscular mycorrhizal fungus. J Plant Physiol 159:1329–1339

Latour X, Lemanceau P (1997) Métabolisme carboné et énergétique des *Pseudomonas* spp. fluorescents saprophytes à oxydase positive. Agronomie 17:427–433

Latour X, Delorme S, Mirleau P, Lemanceau P (2003) Identification of traits implicated in the rhizosphere competence of fluorescent pseudomonads: description of a strategy based on population and model strains studies. Agronomie 23:397–405

Lemanceau P, Corberand T, Gardan L, Latour X, Laguerre G, Boeufgras J M and Alabouvette C (1995) Effect of two plant species, flax (*Linum usitatissimum* L) and tomato (*Lycopersicon esculentum* Mill), on the diversity of soilborne populations of fluorescent pseudomonads. Appl Environ Microbiol 61:1004–1012

Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. Phytopathology 78:366–371
Linderman RG (2000) Effects of mycorrhizas on plant tolerances to diseases. In: Kapulnik Y, Douds DD (eds) Arbuscular mycorrhizas: physiology and function. Kluwer, Dordrecht, pp 345–365
Lingua G, Sgorbati S, Citterio A, Fusconi A, Trotta A, Gnavi E, Berta G (1999) Arbuscular mycorrhizal colonization delays nucleus senescence in leek root cortical cells. New Phytol 141:161–169
López-Bucio J, Cruz-Ramírez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 6:280–287
Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490.
Malamy JE (2005) Intrinsic and environmental response pathways that regulate root system architecture. Plant Cell Environ 28:67–77
Maloney PE, van Bruggen AHC, Hu S (1997) Bacterial community structure in relation to the carbon environments in lettuce and tomato rhizospheres and in bulk soil. Microbial Ecol 34:109–117
Maron P-A, Ranjard L, Mougel C, Lemanceau P (2008) Metaproteomics: a new approach for studying functional microbial ecology. Microb Ecol (in press)
Marsh JF, Schultze M (2001) Analysis of arbuscular mycorrhizas using symbiosis-defective plant mutants. New Phytol 150:525–532
Mayo K, Davis RE, Motta J (1986) Stimulation of germination of *Glomus versiforme* by spore-associated bacteria. Mycologia 78:426–431
Meyer JR, Linderman RG (1986a) Response of subterranean clover to dual inoculation with vesicular-arbuscular mycorrhizal fungi and a plant growth-promoting bacterium, *Pseudomonas putida*. Soil Biol Biochem 18:185–190
Meyer JR, Linderman RG (1986b) Selective influence on populations of rhizosphere or rhizoplane bacteria and actinomycetes by mycorrhizas formed by *Glomus fasciculatum*. Soil Biol Biochem 18:191–196
Mougel C, Offre P, Ranjard L, Corberand T, Gamalero E, Robin C, Lemanceau P (2006) Dynamic of the genetic structure of bacterial and fungal communities at different development stages of *Medicago truncatula* Gaertn. cv. Jemalong line J5. New Phytol 170:165–175
Mucciarelli M, Scannerini S, Bertea CM, Maffei M (2002) An ascomycetous endophyte isolated from *Mentha piperita* L.: biological features and molecular studies. Mycologia 94:28–39
Nagahashi G, Douds DD (2000) Rapid and sensitive bioassay to study signals between root exudates and arbuscular mycorrhizal fungi. Biotechnol Techn 13:893–897
Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23:375–396
Normander B, Hendriksen NB, Nybroe O (1999) Green fluorescent protein marked *Pseudomonas fluorescens*: localization, viability and activity in the natural barley rhizosphere. Appl Environ Microbiol 65:4646–4651
Offre P, Pivato B, Siblot S, Gamalero E, Corberand T, Lemanceau P, Mougel C (2007) Identification of bacterial groups preferentially associated with mycorrhizal roots of *Medicago truncatula*. Appl Environ Microbiol 73:913–921
Oláh B, Brière C, Bécard G, Dénarié J, Gough C (2005) Nod factors and a diffusible factor from arbuscular mycorrhizal fungi stimulate lateral root formation in *Medicago truncatula* via the DMI1/DMI2 signalling pathway. Plant J 44:195–207
O'Sullivan DJ, O'Gara F (1992) Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol Rev 56:662–676
Paszkowski U, Jakovleva L, Boller T (2006) Maize mutants affected at distinct stages of the arbuscular mycorrhizal symbiosis. Plant J 47:165–173
Paulitz TC, Linderman RG (1989) Interactions between fluorescent pseudomonads and VA mycorrhizal fungi. New Phytol 113:37–45
Pinior A, Wyss U, Piché Y, Vierheilig H (1999) Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. Can J Bot 77:891–897

Pozo MJ, Slezack-Deschaumes S, Dumas-Gaudot E (2002) Plant defense responses induced by arbuscular mycorrhizal fungi. In: Gianinazzi S, Schuepp H (eds) Mycorrhizal technology: from genes to bioproducts -achievement and hurdles in arbuscular mycorrhizal research. Birkhauser, Basel, pp 103–112

Pozo MJ, Van Loon LC, Pieterse CMJ (2005) Jasmonates - signals in plant-microbe interactions. J Plant Growth Regul 23:211–222

Raaijmakers JM, Leeman M, van Oorschot MMP, Van der Sluis I, Schippers B, Bakker PAHM (1995) Dose-response relationships in biological control of fusarium wilt of radish by *Pseudomonas* spp. Phytopathology 85:1075–1081

Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81:537–547

Ravnskov S, Jakobsen I (1999) Effects of *Pseudomonas fluorescens* DF57 on growth and P uptake of two arbuscular mycorrhizal fungi in symbiosis with cucumber. Mycorrhiza 8:329–334

Redecker D, Kodner R, Graham LE (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921

Remy W, Taylor TN, Hass H, Kerp H (1994) 4-hundred-million-year-old vesicular-arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Rillig MC, Mummey DL, Ramsey PW, Klironomos JN, Gannon JE (2006) Phylogeny of arbuscular mycorrhizal fungi predicts community composition of symbiosis-associated bacteria. FEMS Microbiol Ecol 57:389–395

Salzer P, Boller T (2000) Elicitor-induced reactions in mycorrhizae and their suppression. In: Podila GK, Douds DD (eds) Current advances in mycorrhizae research. American Phytopathological Society Press, St. Paul, Minn., pp 1–10

Salzer P, Bonanomi A, Beyer K, Vogeli-Lange R, Aeschbacher RA, Lange J, Wiemken A, Kim D, Cook DR, Boller T (2000) Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. Mol Plant Microbe Interact 13: 763–777

Scannerini S, Fusconi A, Mucciarelli M (2002) The effect of endophytic fungi on host plant morphogenesis. In Seckbach J (ed) Symbiosis, cellular origin and life in extreme habitats. Kluwer, Dordrecht, pp 427–447

Schippers B, Bakker AW, Bakker PAHM (1987) Interactions of deleterious and beneficial rhizospheric microorganisms and the effect on cropping practices. Annu Rev Phytopathol 25:339–358

Selosse MA, Le Tacon F (1998) The land flora: a phototroph-fungus partnership? Trends Ecol Evol 13:15–20

Semenov AM, van Bruggen AHC, Zelenev VV (1999) Moving waves of bacterial populations and total organic carbon along roots of wheat. Microb Ecol 37:116–128

Shaul-Keinan O, Gadkal V, Ginzberg I, Grunzweig JM, Chet I, Elad Y, Wininger S, Belausov E, Eshed Y, Arzmon N, Ben-Tal Y, Kapulnik Y (2002) Hormone concentrations in tobacco roots change during arbuscular mycorrhizal colonization with *Glomus intraradices*. New Phytol 154:501–507

Simons M, van der Bij AJ, Brand I, de Weger LA, Wijffelman CA, Lugtenberg BJJ (1996) Gnotobiotic system for studying rhizosphere colonization by plant growth promoting *Pseudomonas* bacteria. Mol Plant Microbe Interact 9:600–607

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Sood SG (2003) Chemotactic response of plant-growth promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol Ecol 45:219–227

Staehelin C, Charon C, Boller T, Crespi M, Kondorosi A (2001) *Medicago truncatula* plants overexpressing the early nodulin gene enod40 exhibit accelerated mycorrhizal colonization and enhanced formation of arbuscules. Proc Natl Acad Sci USA 98:15366–15371

Sthal E (1900) Der Sinn der Mycorhizenbildung. Jahrb Wiss Bot 34:539–668

Strack D, Fester T (2006) Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. New Phytol 172:22–34

Strigul NS, Kravchenko LV (2006) Mathematical modelling of PGPR inoculation into the rhizosphere. Environ Modell Softw 21:1158–1171

Stumpe M, Carsjens J-G, Stenzel I, Gobel C, Lang I, Pawlowski K, Hause B, Feussner I (2005) Lipid metabolism in arbuscular mycorrhizal roots of *Medicago truncatula*. Phytochemistry 66:781–791

Tamasloukht MB, Séjalon-Delmas N, Kluever A, Jauneau A, Roux C, Becard G, Franken P (2003) Root factors induce mitochondrial-related gene expression and fungal respiration during the development switch from symbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora margarita*. Plant Physiol 131:1468–1478

Tawaraya K, Watanabe S, Yoshida E, Wagatsuma T (1996) Effect of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. Mycorrhiza 6:57–59

Tisserant B, Schellenbaum L, Gianinazzi-Pearson V, Gianinazzi S, Berta G (1992) Influence of infection by an endomycorrhizal fungus on root development and architecture in *Platanus acerifolia*. Allionia 30:173–183

Tisserant B, Gianinazzi S, Gianinazzi-Pearson V (1996) Relationships between lateral root order, arbuscular mycorrhiza development, and the physiological state of the symbiotic fungus in *Platanus acerifolia*. Can J Bot 74:1947–1955

Tombolini R, Van der Gaag DJ, Gerhardson B, Jansson JK (1999) Colonization pattern of the biocontrol strain *Pseudomonas chlororaphis* MA342 on barley seeds visualized by using green fluorescent protein. Appl Environ Microbiol 65:3674–3680

Torelli A, Trotta A, Acerbi L, Arcidiacono G, Berta G, Branca C (2000) IAA and ZR content in leek (*Allium porrum* L.), as influenced by P nutrition and arbuscular mycorrhizae, in relation to plant development. Plant Soil 226:29–35

Toth R, Miller RM (1984) Dynamics of arbuscule development and degeneration in a *Zea mays* mycorrhiza. Am J Bot 71:449–460

Unge A, Jansson J (2001) Monitoring population size, activity and distribution of gfp-luxAB tagged *Pseudomonas fluorescens* SBW25 during colonization of wheat. Microb Ecol 41:290–300

Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Ann Rev Plant Physiol 26:453–483

Van Peer R, Punte HLM, de Weger LA, Schippers B (1990) Characterization of root surface and endorhizosphere pseudomonads in relation to their colonization of roots. Appl Environ Microbiol 56:2462–2470

van Veen JA, van Overbeek LS, van Elsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol Rev 61:121–135

Varney GT, McCully ME (1991) The branch root of *Zea*. II. Developmental loss of the apical meristem in field grown roots. New Phytol 118:535–546

Vasquez MM, Cesar R, Azcon R, Barea JM (2000) Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (*Azospirillum, Pseudomonas, Trichoderma*) and their effects on microbial populations and enzyme activities in the rhizosphere of maize plants. Appl Soil Ecol 15:261–272

Vierheilig H (2004a) Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Bot 82:1166–1176

Vierheilig H (2004b) Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. J Plant Physiol 161:339–341

Vierheilig H, Piche Y (2002) Signalling in arbuscular mycorrhiza: facts and hypotheses. Adv Exp Med Biol 505:23–39

Vierheilig H, Alt M, Mohr U, Boller T, Weimken A (1994) Ethylene biosynthesis and β-1,3-glucanase in the roots of host and non-host plants of vesicular-arbuscular mycorrhizal fungi after inoculation with *Glomus mosseae*. J Plant Physiol 143:337–343

Vierheilig H, Garcia-Garrido JM, Wyss U, Piché Y (2000) Systemic suppression of mycorrhizal colonization of barley roots already colonized by AM fungi. Soil Biol Biochem 32:589–595

Vierheilig H, Lerat S, Piche Y (2003) Systemic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonized by *Glomus mosseae*. Mycorrhiza 13:167–170

Volpin H, Elkind Y, Okon Y, Kapulnik Y (1994) A vesicular arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defense response in alfalfa roots. Plant Physiol 104:683–689

Vosatka M., Gryndler M (1999) Treatment with culture fractions from *Pseudomonas putida* modifies the development of *Glomus fistulosum* mycorrhiza and the response of potato and maize plants to inoculation. Appl Soil Ecol 11:245–251

Walker TS, Bais HP, Grotewold E, Vivanco JM (2003) Root exudation and rhizosphere biology. Plant Physiol 132:44–51

Walsh UF, Morrisey JP, O'Gara F (2001) Pseudomonads for biocontrol of phytopathogens: from functional genomics to commercial exploitation. Curr Opin Biotechnol 12:289–295

Watnick P, Kolter R (2000) Biofilm, city of microbes. J Bacteriol 182:2675–2679

Weller DM (1983) Colonization of wheat roots by a fluorescent pseudomonad suppressive to take-all. Phytopathol 73:1548–1553

Weller DM (1988) Biological control of soilborne plant pathogen in the rhizosphere with bacteria. Annu Rev Phytopathol 26:379–407

Weller DM, Thomashow LS (1994) Current challenges in introducing beneficial microorganisms into the rhizosphere. In: O'Gara F, Dowling DN, Boesten B (eds) Molecular ecology of rhizosphere microorganisms. VCH, Weinheim, pp 1–18

Whipps JM (1997) Developments in the biological control of soilborne plant pathogens. Adv Bot Res 26:1–133

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Xie Z-P, Staehelin C, Vierheilig H, Wiemken A, Jabbouri S, Broughton WJ, Vögeli-Lange R, Boller T (1995) Rhizobial nodulation factors stimulate mycorrhizal colonization of nodulating and nonnodulating soybeans. Plant Physiol 108: 1519–1525

Xie Z-P, Müller J, Wiemken A, Broughton WJ, Boller T (1998) Nod factors and tri-iodobenzoic acid stimulate mycorrhizal colonization and affect carbohydrate partitioning in mycorrhizal roots of *Lablab purpureus*. New Phytol 139: 361–366

Zaidi A, Khan MS (2005) Interactive effect of rhizotrophic microorganisms on growth, yield, and nutrient uptake of wheat. J Plant Nutr 28: 2079–2092

In vitro Cultures Open New Prospects for Basic Research in Arbuscular Mycorrhizas

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1 Introduction

1.1 Arbuscular Mycorrhizas In Vitro Cultures: The Starting Material

It is now 20 years since the publication of Bécard and Fortin's article (1988) in which the modern basis for arbuscular mycorrhizal (AM) monoxenic cultures was established. Since then, many research projects have been carried out using in vitro systems, and new prospects have been opened up by utilizing the amazing research material provided by monoxenic plates. More and more researchers now acknowledge that in vitro AM production is the sole way of getting large amounts of clean, clonal, contamination-free AM fungal material. This has opened the doors for molecular biology and biochemical techniques to be applied to mycorrhizal research, and the direct consequence of this is an exponential increase in our knowledge in the basic biology of this mutualistic symbiosis over the last 10 years. However, monoxenic cultures have far more to offer than just being AM tissue factories; fields of AM research as different (yet interlinked) as colony architecture and dynamics, intra- and extraradical fungal morphology, mycorrhizal physiology, biotic and abiotic stress responses, microbial interactions and even the production of ultrapure, mycorrhiza-based biofertilizers have benefited from this 'in vitro revolution'.

This chapter aims to summarize the opportunities offered by using in vitro culture technology, and to encourage researchers to (1) utilize existing, yet little known monoxenic culture techniques, and (2) improve them and design new in vitro experimental systems. It should not be regarded as a compilation of methods, but rather as a brainstorming exercise designed to create new avenues of mycorrhizal research.

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1.2 Starting a New Arbuscular Mycorrhizal Monoxenic Culture (AMMC): The Real Challenge

Research using monoxenic cultures in the late 1980s began mainly with using *Gigaspora margarita* DAOM 194757 (Bécard and Fortin 1988; Bécard and Piché 1989a, 1989b; Chabot et al. 1992a). From the 1990s, the AM fungal isolate, *Glomus intraradices* DAOM 197198 (Chabot et al. 1992b), was mostly used. This was mainly due to the fact that this AMF isolate is well-adapted to the monoxenic culture conditions, producing important amounts of tissue and being quite easy to replicate. As a consequence of this, *G. intraradices* DAOM 197198 has become a kind of 'model AMF species', comparable to the model plant *Arabidopsis thaliana* or bacteria *Escherichia coli*. It was plain from the beginning, however, that diversification using and comparing different AMF isolates/species/genera was mandatory in order to have a global and realistic view of mycorrhizal symbiosis.

The difficulties of introducing some AMF isolates/species into monoxenics lead some researchers to claim this system as 'artificial', although new techniques have recently been described which can resolve this issue (see Section 3.3). Nevertheless, monoxenic cultures have gone ahead, and in the last 5 years there has been an increase in the availability of different species and isolates and a reasonable number of scientific studies published (see St-Arnaud 2005 for listing). The first exclusively in vitro collection of AMF [Glomeromycota IN vitro Collection (GINCO)] has been created and is jointly hosted in Canada and Belgium (*http://emma.agro.ucl.ac.be/ginco-bel/*; *http://res2.agr.gc.ca/creco/ginco-can*). To date, it comprises of around 20 different species/isolates from all around the world. Some other institutions also maintain AMF in vitro collections either for research (e.g., Ian Sanders, personal communication; A. Bago, personsal communication) or for commercial purposes (e.g., MYCOVITRO S.L., Granada, Spain).

Initialization of a new AM monoxenic culture is theoretically an easy process, yet in practice is tricky and difficult to achieve for many. Spores can be isolated from rhizospheric soils of interest using the process of wet-sieving and decanting. If there is an abundance of healthy-looking AMF spores then processing can continue. In soils where AM spore numbers are low, establishing trap plant cultures may become necessary to increase the spore population. This may take several months of plant growth. In either case, the results should be to get as many viable spores as possible and to have them free of soil debris. To achieve this, selection of spores one by one with special flexible forceps is highly recommended. If recognition of the different morphotypes among the isolated spores is easy and feasible, the next step would be to group them and to surface-sterilize group by group, thereby giving the highest guarantee of success for initiating a new monoxenic culture. In fact, a synergism in spore germination has been frequently noted, thus maximizing possibilities of obtaining host root colonization (A. Bago, personal observation). Unfortunately, however, it is not often easy to differentiate soil-collected spores into distinct isolate groupings, so that this straightforward path is usually only applicable in cases where a pot culture of a previously isolated pure AMF species has been used. Therefore,

generally speaking, we could say that monosporic monoxenic cultures are mandatory. To do this, the whole spore population should be surface-sterilized, and subsequently platted one by one (flexible forceps strongly recommended) into separate plates containing the appropriate monoxenic culture medium (for protocols, see Cranenbrouck et al. 2005; Declerck et al. 2005).

Methods of surface-sterilizing AM spores are among the first techniques that young mycorrhizologists learn, and the most accepted one is using the Kitasato beaker method (Mosse 1962). Briefly, this consists of using the flask coupled to a filter device to maintain spores in continuous suspension within a sterilization solution (comprising of chloramine T, a mixture of generalist antibiotics and traces of a surfactant agent), then applying a vacuum to eliminate the solution and proceeding to wash with sterile water. The whole procedure is conducted in sterile conditions using a laboratory hood. This traditional sterilization procedure has now been recently improved by the Eppendorf surface sterilization method (ESSM) (Cano et al., unpublished). This method substitutes the Kitasato flask and vacuum system with a simple Eppendorf tube with the spores in the sterilizing solution. The ESSM has the advantage of being less complicated to manipulate, requiring much less sterilizing solution, and avoiding the inconveniences of recovering the spores from the filter paper, thus minimizing the loss of spores during the process.

Once surface-sterilized and platted, individual spores are left to germinate, after which they are put into contact with an appropriate host root to induce mycorrhization. Here we reach the core of the in vitro cultivation techniques of AM, since these two steps are, unfortunately, the ones over which we have least control. Some studies have investigated the issue of inducing the germination of spores (e.g., Hepper 1984; Juge et al. 2002), but the process of germination is still unknown, and apparently some healthy viable-looking spores do not germinate even after cold treatments. Results in our laboratory indicate to a possible seasonality of spores which seems to affect some genera more (i.e., *Gigaspora*) than others (i.e., *Glomus*). In *Gigaspora*, the seasonality is quite remarkable and, according to preliminary data, spores do not tend to germinate in September whereas germination is quite common around February (Cano and Bago, unpublished). This is even more surprising given the fact that in both cases spores were maintained under highly controlled in vitro conditions with constant light and temperature; it would be tempting to speculate that biological clocks are at work in AM fungal spores. Clearly, much more research is needed on this crucial subject.

We also have still much to understand about the processes which induce an AM fungus to penetrate the root and establish symbiosis at a precise site and moment (Vierheilig and Bago 2005). The fact that a continuous nuclear migration along fungal mycelium occurs (Bago et al. 1998b, 1999a; Giovannetti et al. 2001), together with the possible heterocaryotic nature of AMF (Sanders 2004), allows us to speculate that compatibility between the host–fungal nuclei at very early stages of symbiosis establishment is at work, and that plant–fungal symbiont's compatibility is a far more complicated process than envisaged.

Once the first serious barrier of starting a new AMMC has been overcome, there is a subsequent process of AM fungal 'taming' or adaptation to the in vitro culture

media. Let us mention three well-known AMF as an example for this. The first one is *Glomus intraradices*, which after being isolated from the soil is usually able to quickly adapt (1–2 replication cycles) to the new in vitro conditions; in fact most of the in vitro currently-maintained isolates belong to this species. *G. intraradices* is recognized as a highly competitive AMF which perhaps explains its adaptability to the aseptic conditions and artificial media. In our laboratory, the same *G. intraradices* isolate has been maintained in continuous culture since 1996. The second example, *Glomus etunicatum*, has been claimed to be maintained in monoxenics in different occasions, although the authors reported little or no sporulation after a few replication rounds (Pawlowska et al. 1999, and personal communication). This fungus certainly shows a different characteristic: it definitely requires very young spores to be subcultured onto fresh medium at and at least 3–5 replication rounds to become 'tamed', thereby taking twice as much time as *G. intraradices* to become successfully cultured in monoxenics. This fungus has now been maintained in continuous culture in our laboratory since 2003. The third example is that of continuous failure: it refers to *Glomus mosseae*, one of the most desired yet reluctant species to get into monoxenics. Different efforts carried out to establish it in culture have ended without success (although sometimes claimed otherwise; e.g., Douds 1997; Fortin et al. 2002). However, expectations were high as the fungus penetrated the root and even formed intraradical and extraradical symbiotic structures (arbuscules and BAS; Cano, personal communication). However, growing stopped shortly after with the production of a few (<10) or no new spores. In other words, we do not know yet how to 'tame' this fungus; is it a question of finding the right host root or culture conditions? Clearly more research is needed on the subject. New culture systems which could help to circumvent this reluctance are described below (Section 3.4).

In summary, starting a new AMMC requires patience, skill and expertise. It will be no big surprise if in a near future, the demand to establish and maintain new/required monoxenic cultures will create specialized institutions or companies. The increase in availability of such cultures will undoubtedly help in widening our knowledge on AM fungal biology.

1.3 The Importance of Host Roots When Studying Mycorrhizal Symbiosis in vitro

It is believed by many that in vitro AM cultures only use transformed roots; that being *Daucus carota* L. (carrot) hairy root cultures. This, however, is not the case, and the use of non-transformed roots, availability of plant root mutants and development of new methods for culturing whole plants in vitro has given increased possibilities and opportunities for future research.

The bases for root organ culture (ROC) were originally developed by White (1934) by obtaining continuous culture of non-transformed tomato (*Solanum lycopersicum* Mill.) roots in a liquid medium. Mosse and Hepper (1975) established the first in vitro

co-culture (on solid media) between a ROC and an AMF (AM monoxenic culture) using excised non-transformed roots of *S. lycopersicum* and *Trifolium pratense* L., and showed that roots were colonized and the fungal life cycle was completed by the production of a few spores. It was not until 1987 that Mugnier and Mosse (1987) suggested the transformed ROC culture technology for AM in vitro culturing; this paper was the basis for that of Bécard and Fortin's (1988). Variations of the culture media by Strullu and Romand (1986; 'MSR medium'), Bécard and Fortin (1988; 'M medium'), Chabot et al. (1992b; 'MW medium') and St-Arnaud et al. (1996; 'M-C medium') have further allowed both transformed and non-transformed ROCs to become mycorrhizated in vitro, and subsequently maintained in continuous culture.

The architecture of transformed roots of *Daucus carota* L. and *Medicago truncatula* has been compared to non-transformed roots and found to be similar (Bécard and Fortin 1988; Boisson-Dernier et al. 2001). However, transformation of roots does appear to influence the development of AM and colonization is often higher with a greater amount of extraradical mycelium being supported than with non-transformed roots (see Müller et al. 1999; Barker and Tagu 2000; Fortin et al. 2002), this probably due to their modified hormone balance. ROCs of a number of *S. lycopersicum* cultivars, both in non-transformed and transformed states, have been used in a number of separated studies. Other plant roots to have been transformed and used in monoxenic cultures include *Linum usitatissimum* L., *Pisum sativum* L. and *Tagetes patula* L. (see Bago and Cano 2005 for full listing). Most of these plants have fine root systems capable of growing on monoxenic plates for a reasonable time period before the need for transferral to new plates; therefore, there seems to be no reason to expect that they would not grow under in vitro conditions without transformation. Side-by-side detailed experiments to investigate the effect of plant transformation on different AM fungi may be a useful exercise to investigate any structural or molecular changes that may occur.

Using transformed ROCs of *Pisum sativum* L. mutants altered in their interaction with *Rhizobium*, Balaji et al. (1994) assessed that AMF behaviour in vitro was similar to that observed under pot conditions. The authors claimed such experimental system as model for testing root susceptibility to mycorrhizal colonization. More recently, the root growth and AM colonization patterns have been compared between the non-transformed wild type *S. lycopersicum* ROC (76R) and a mutant line derived from it exhibiting a highly *r*educed *m*ycorrhizal *c*olonization phenotype (*rmc*) in both monoxenic cultures and in soil (Bago et al. 2006). No differences were observed between 76R and *rmc* genotypes in terms of root length, number of cortical cell layers, phenolic deposition in the outer cell layers of the roots or in the structure of the xylem, or in the structural root autofluorescence in both monoxenics and soil (Fig. 1). The only significant differences in root anatomy for monoxenic versus soil culture were that monoxenic roots had significantly smaller air spaces and increased starch deposits (Bago et al. 2006). All these results suggest that, by making use of the controlled uniform conditions produced for monoxenic culture systems, there are many possibilities of using plant mutants in vitro to investigate both the characteristics of rhizobial nodulation and AM colonization.

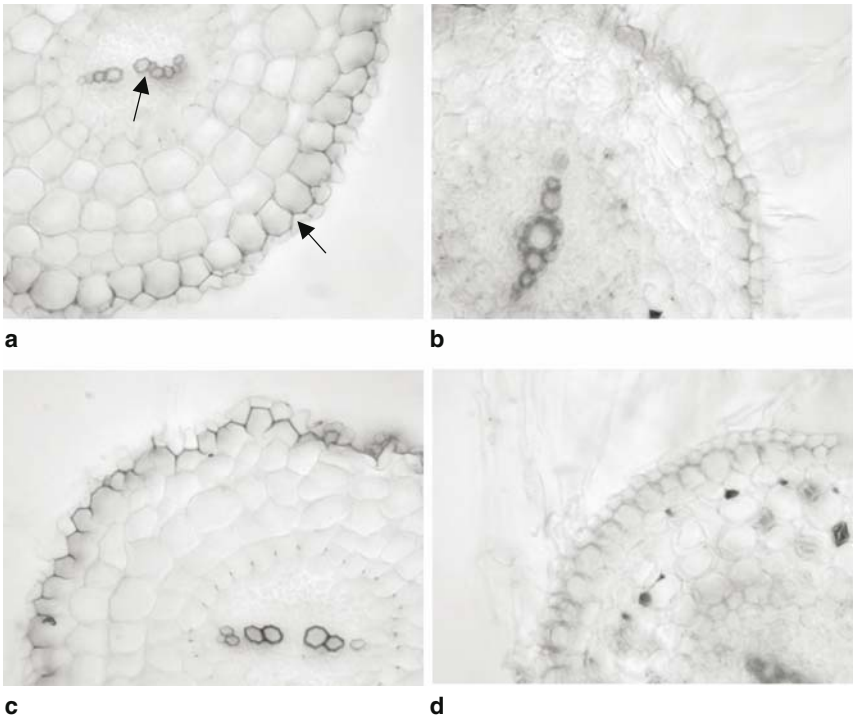

Fig. 1 Transverse sections of diarch tomato (*Solanum esculentum* L.) roots (cvs.76R and *rmc*). Staining with phloroglucinol/HCl indicated that phenolic deposition in the outer cell layers of the roots and in the xylem (*arrows*) was similar between both genotypes and growing conditions. **a** 76R, soil-grown roots; **b** 76R, monoxenically-grown roots; **c** *rmc*, soil-grown roots; **d** *rmc*, monoxenically-grown roots. Photos: Dr. Sally Smith

ROC systems, until recently, have all been separated from the host shoot and grown on M or MW medium supplying carbon (C). Whole plant in vitro systems have now been established in which either the roots or the entire plant itself is grown under aseptic conditions (Voets et al. 2005; Dupré de Boulois et al. 2006; see Section 3.3). These whole-plant new methods overcome any controversy or questionability that has previously arisen due to the use of using only roots as hosts (Bago 1998; Bago and Cano 2005).

Another interesting issue with the selection of the host root for in vitro experiments is the type of morphological development the AM fungus expresses when colonizing that root, and whether or not this affects or alters either the plant or fungal metabolism or their gene expression. Distinct structural classes within AM symbioses (*Arum*-type and *Paris*-type, and a number observed inbetween them) were described by Gallaud (1904, 1905). *Arum*-type morphology consists of mycelium which spreads along the plants cortical intercellular airspaces and penetrates the cortical cells to form individual terminal intracellular

arbuscules, whereas in the *Paris*-type the fungus is entirely intracellular, forming irregular coiled mycelium and arbusculate coils. Gallaud (1905) stated that the morphology of arbuscular mycorrhiza was influenced both by the host and the fungal species. It has been shown that one AM fungus can form either type of colonization depending upon the host plant, and some plants appear to produce strictly only one morphological type (see Smith and Smith 1997, for references therein). However, fungal identity does also play a role and some plants can produce either *Arum*- and *Paris*-types of AM (as in the case of *S. lycopersicum*) or a range of morphological types with different AM fungi (Cavagnaro et al. 2001; Dickson 2004).

It is not clear to the extent that these morphological differences can influence the physiology and functional compatibility of the symbiosis. Positive growth responses and improved phosphate (P) nutrition have been shown in many plant species forming *Arum*-type AM (see Smith and Smith 1997). It has also been shown in soil-grown plants that *Paris*-type coils can transfer P to *M. truncatula* (Smith et al. 2004). Plants with coils and arbusculate coils and several plant species with transformed roots showing intermediate AM morphology also have AM-inducible plant P transporters (Karandashov et al. 2004; Glassop et al. 2005). However, defence reactions by the host towards the fungus do vary between the morphological types. Defence-related genes increased in *S. lycopersicum* when colonized by *S. calospora* (which formed *Paris*-type structures), in contrast to two isolates of *G. intraradices* (forming *Arum*-type structures) in which they were suppressed (Gao 2002; Gao et al. 2004). The authors suggested that this higher expression might be related to the extensive penetration of the cortical cell walls in *Paris*-type AM. If true, this would be important to take into account when doing AM gene expression studies. This additional morphological/functional complexity of AM interactions could be certainly studied in depth by using monoxenic systems.

1.4 Continuous in vitro Culture of AM to Obtain Pure, Clonal Mycorrhizal Tissues

In Section 1.1 we have discussed the challenge of getting a given AM fungus 'tamed' so that it develops and fully completes its life cycle in vitro. An additional challenge when cultivating monoxenically consists of maintaining such interactions over generations, i.e., to get the continuous monoxenic culture. Although frequently unnoticed, this is a crucial issue which would (1) ensure the correct functioning of the in vitro symbiotic interaction, and (2) allow clonal fungal material, essential to carry out molecular studies, to be obtained while minimizing undesirable external variation.

The main example of successful continuous AM monoxenic culture is (again) *G. intraradices* DAOM 197198, which has been maintained continuously since 1992 (and probably before; Chabot et al. 1992). During these 15

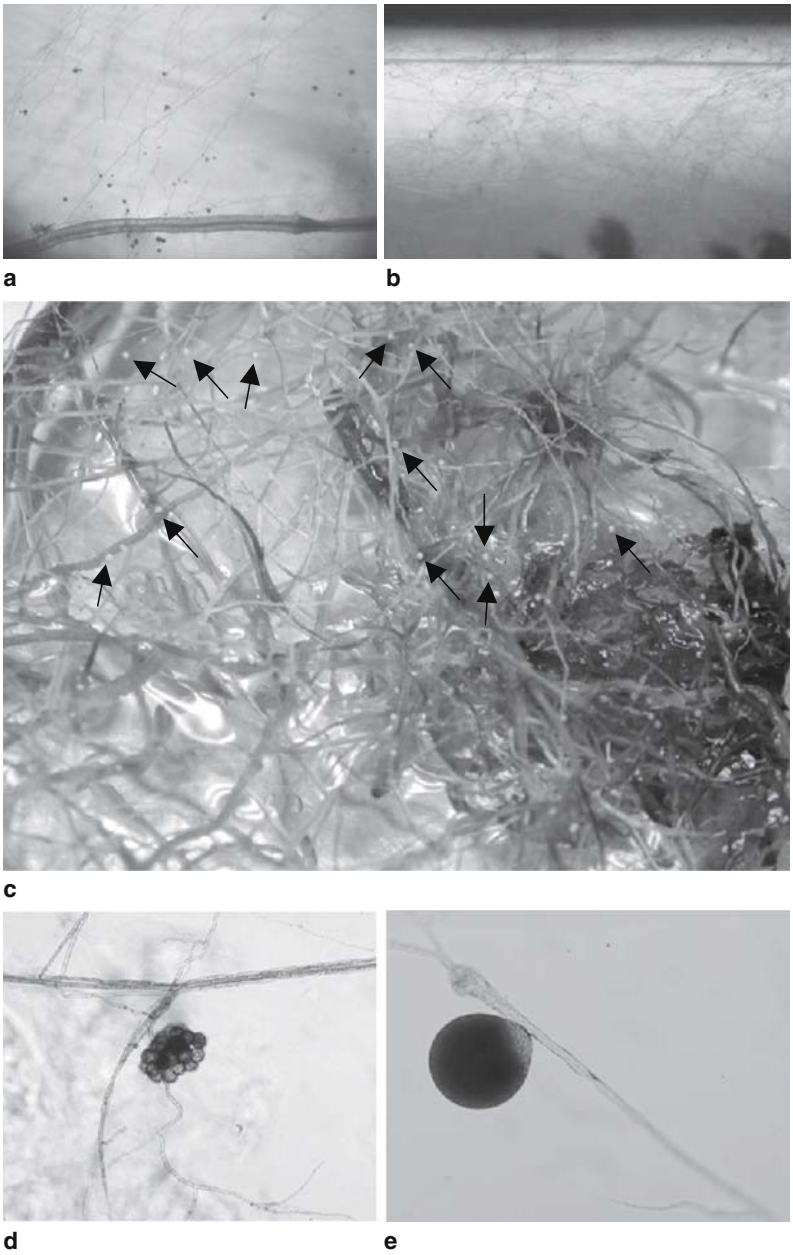

Fig. 2 Continuous in vitro culture of *Glomus etunicatum* B-1 (CIMA-07, **a,b**) or *Gigaspora margarita* BEG-34 (CIMA-04, **c**–**e**). Details of colony architecture (**a,b**), auxiliary cells and spore formation (**d,e**) can be observed in situ due to the monoxenic transparent culture medium. **c** A general view of a *Gigaspora margarita* plate with numerous newly-produced spores (some of them indicated by *arrows*)

years of continuous subculturing (meaning over 60 generations if calculating on 4 generations a year) the fungus has established successful AM symbioses with several different host ROCs (most commonly being with *D. carota* DC-1 and DC-2 clones, and *S. lycopersicum* cultivars Vendor, 76R and *rmc*), and producing a mean of 30.000 spores per Petri plate. It has never shown signs of loss of viability or decadency over this time; a highly remarkable feat indeed for a supposedly lower, asexual fungus.

Other successful examples of continuous in vitro culturing include the *G. etunicatum* referred to in Section 1.1. Although difficult to 'tame', once adapted to the in vitro conditions it responds reasonably well, and it has now been maintained under continuous culture for 4 years (i.e., over 16 generations) with an estimated mean production of 2.000 spores per plate (Fig. 2a,b). The third example is *Gigaspora margarita* BEG-34, a species that many still believe impossible to subculture, which has been maintained in our laboratory since 2000, with over 32 successive generations and producing around 50 new spores per plate (Cano and Bago, unpublished) (Fig. 2c–e).

A number of other reported monoxenic cultures have not been maintained over time, (meaning those that were once *really* monoxenic cultures; see Cano and Bago 2005). To the best of our knowledge, none of those losses were due to exhaustion of the fungal inoculum. Maintenance of AM monoxenic cultures requires expert skills and dedication, which is sometimes incompatible with funds allocated to research groups. Some interesting monoxenic cultures previously used are no longer available, such as *Glomus caledonium* (Karandashov et al. 2000), *G. versiforme* (Plenchette et al. 1996) or the *Gigaspora margarita* described in the Bécard and Fortin (1988) paper. Hopefully growing awareness of the importance of maintaining and increasing AM in vitro germplasm collections will avoid these terrible loses to happen again.

2 The Use of Monoxenic Cultures in Basic AM Research: Advantages, Drawbacks and Future Challenges

Any biochemical or molecular study of the biology of a given organism requires pure material, easily obtainable and in quantities large enough to extract the biomolecules of interest. Furthermore, when studying organisms which develop in a very heterogeneous environment such as soil, a consensus in the culture conditions is required in order to compare independent results from different laboratories. These requirements were seriously restricted before the development of AMF in vitro cultures. Monoxenic cultures have enabled researchers worldwide to obtain fungal material grown under the same conditions, with an extreme degree of purity, free of undesired contamination and in quantities large enough to exponentially increase our understanding of the molecular and biochemical processes of the Glomeromycota.

2.1 Using in vitro Cultures to Study Environmental Conditions Affecting Arbuscular Mycorrhizas

There is no doubt that despite the enormous possibilities the system offers, in vitro monoxenic cultures are artificial devices which try to imitate conditions encountered by mycorrhizal roots in nature. Yet, as it has been the case with the rest of in vitro cultures (from bacteria, to human stem cell cultures), these systems allow us to control environmental conditions, and so to study fungal behaviour under known parameters.

As an example of this, monoxenic cultures have been used to study the influence of temperature on root and fungal development, and to relate this with C acquisition by the fungus, and therefore to symbiotic efficiency. Gavito et al. (2005) grew monoxenic plates of two *Glomus* species (*G. intraradices* GINCO/MUCL 43194) and *G. proliferum* GINCO/MUCL 41827) which were maintained at temperatures ranging from 6° to 30 °C. The results obtained clearly showed that temperatures between 6° and 18 °C reduced AMF growth, and a reduction in C translocation to the fungus was evident. A clear effect on root development was also observed at high temperatures (28° and 30 °C) with roots darkening and growth stopping (Fig. 3a) while the AM fungus was less (or not) affected. The optimum temperature for both root and fungus was 24 °C, which is the one utilized in standard AM in vitro culture protocols.

Monoxenic cultures have also helped us to better understand the effect of AMF development on substrate pH. The combined use of monoxenic cultures and the pH indicator bromocresol purple revealed that strong acidification of the substrate takes place in areas where either ammonium is being taken up (Villegas et al. 1996) or a massive fungal sporulation occurred (Fig. 3b) (Bago et al. 1998). A clear acidification of the culture media was also observed surrounding mycorrhizal subapical zones. When nitrate was provided as the sole N source however, strong alkalinization of the culture media occurred in areas surrounding actively nutrient-acquiring roots (Bago et al. 1996).

2.2 Microscopy of AM Monoxenic Cultures: Fungal Biology at a Glance

Morphogenetic and cellular studies of AM using in vivo microscopy can benefit from using AMMC due to the transparent culture medium and the easiness to follow fungal development over time (Bago et al. 1998a). However, there is a challenge to develop methods for the adequate staining and observation of colonization in plant roots and fungal extraradical mycelium development.

Staining of ROCs in situ is not a very effective system as most roots require clearing with potassium hydroxide prior to staining. Covering the Petri plates with stain, leaving for a period of time and rinsing will only partly stain root material

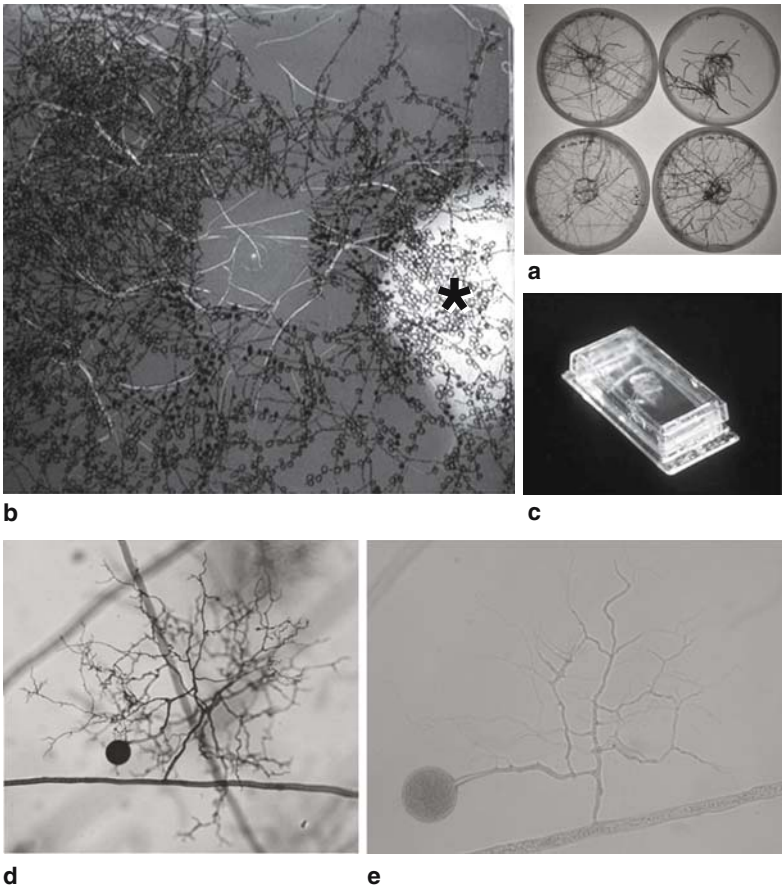

Fig. 3 Use of AM monoxenic cultures for physiological and cellular studies. **a** Temperature tests using monoxenic cultures of DC2+ *Glomus proliferum* (*upper row*) or *G. intraradices* (*lower row*). Plates in the *left column* were incubated at 24 °C and show vigorously growing cultures. Those in the *right column* were maintained at 30 °C, provoking darkening of host roots. Photo: Dr. Mayra Gavito. **b** A 30-cm^2 Petri plate containing an AM monoxenic culture of *Daucus carota* DC2-ROC and *G. intraradices* DAOM197198. The culture medium contains the pH dye bromcresol purple, which turns yellow in acidic zones as the one shown by the asterisk (*). To better visualize, ERM development has been highlighted with a permanent marker, and spores represented by *dots*. **c** a chambered coverslip system to do microscopy in AM monoxenics at high magnification. **d,e** Comparison of in situ staining of an AM monoxenic plate with either trypan blue (*left*) or acid fuschin (*right*) showing the idoneity of the former over the latter on a BAS-s

which is not adequate enough for assessing the level of colonisation (C. Cano, personal observation). Therefore, the most practical method for staining roots is to remove them from the monoxenic plate beforehand. This generally involves liquefying the culture medium with sodium citrate (Doner and Bécard 1991) for easy removal of the mycorrhizal tissues. An alternative procedure for this is to remove

roots one by one with forceps. However, ROCs are usually extremely fragile, so that this is not recommended. Once free of the medium the staining procedure can then be the same as that normally used with staining roots for observation under light or fluorescence microscopy. The most commonly used non-vital stain for light microscopy has been that using a solution containing trypan blue (Phillips and Hayman 1970) with modification to remove some of the toxic reagents from the mixture (Koske and Gemma 1989). Other stains also include acid fuchsin (Kormanik and McGraw 1982), chlorazol black E (Brundrett et al. 1984), aniline blue (Grace and Stribley 1991) and the increasingly used non-toxic solution of ink and vinegar (Vierheilig et al. 1998).

The staining of the extraradical mycelium (ERM) and branched absorbing structures (BAS), however, is trickier, yet possible. If you wish to keep the integrity of the structures (especially the fine BAS) then staining must be conducted in situ. Staining can be performed on a whole plate scale for low magnification assessment, or if higher magnification is needed, the use water immersion objectives directly upon the culture medium (Bago et al. 1999a) or special chambered coverslips to establish the monoxenic culture (Fig. 3c) can be employed.

Stains such as trypan blue can be used for light microscopy (Fig. 3d). Staining times will be longer than that required for roots, and afterwards the plates must be thoroughly rinsed with water until the medium appears transparent, as, if unwashed, the medium absorbs the dye too strongly so contrast is poor. Once rinsed, observation of fungal structures is possible. Staining with acid fuchsin or ink and vinegar does not work very well, as the colour of the stain tends to either remain pale and hence difficult to see, or it does not even penetrate the medium to reach the ERM or BAS. (S. Dickson, unpublished; Fig. 3e). Obviously, testing of other stains would be useful in order to find those that produce well stained fungal structures under different media conditions. Successful staining in situ depends of course upon both the composition of the stain and the medium in which it is used. It is highly possible that some stains previously used for staining roots will react differently to the pH of the medium or to the ingredients within it.

Staining of ERM and BAS for observation with fluorescence and laser scanning confocal microscopy (LSCM) or multi-photon microscopy requires using fluorochromes that can penetrate the medium without producing too much background fluorescence within it. For example, acridine orange instantly stains the medium and this is not removed by washing in water, therefore leaving it impossible to visualize fungal structures (S. Dickson, unpublished). Acid fuchsin has been previously used for visualising AM structures within plants roots with LSCM (Dickson and Kolesik 1999; Dickson et al. 2003). However, the small diameter and fine structure of the BAS branches tend to limit the amount of fluorescence produced, and images can appear patchy. Although this may not be a problem with general imaging, if 3D reconstruction and measurement of surface area or volume are desired then this patchiness may well present a problem. For LSCM the staining of BAS in situ requires further investigation.

The last issue to consider with observation in situ is the depth of the medium itself and the fact that it is also going to play a role in the imaging, whether it is

using light microscopy or confocal microscopy. As mycelium grows through the medium it can travel in three dimensions and the BAS produced also form in three dimensions. This adds extra complexity to those trying to visualise and record these structures. The ability to visualize structures in deep tissue (or medium) relies on being able to penetrate through the medium and this requires a microscope objective with a long working distance. The imaging of ERM and BAS using the LSCM may be difficult as the scattering of the confocal excitation and emission can preclude sharp optical slicing. This is less of a problem with using the multi-photon as penetration into tissue is better. If using either the LSCM or the multi-photon for 3D measurement, refractive index mismatch between the immersion medium of the objective lens used and the culture medium containing the BAS, may result in the overestimation of the actual depth of tissue visualised (White et al. 1996). This axial distortion between the optical slices must be corrected if true measurements of the structures are to be obtained.

The multi-photon can penetrate well into the monoxenic culture medium and this has been shown in the application of imaging fungal nuclei within germ-tubes of *Gigaspora rosea* (Bago et al. 1998b) or the extraradical mycelium of *Glomus intraradices* (Bago et al. 1999a) using DAPI as stain. This technique coupled with Nile red stain also allows the observing of the movement of lipid bodies through the fungal colony of both *G. intraradices* and *Gigaspora margarita* and to determine their volumes, distribution, and velocities (Bago and Bécard 2002; Bago et al. 2002).

Overall we now have some methods for imaging ERM and BAS in the in vitro systems. Clearly more work needs to be conducted in optimising these techniques particularly if results are to be used for surface area or volume measurements.

2.3 Compartmentalized in vitro Systems: A Step Forward in the Study of AM Physiology, Gene Expression and Microbial Interactions

2.3.1 Physiology and Gene Expression

The first physiological studies carried out using monoxenic cultures addressed the influence of root exudates in symbiosis establishment in an attempt to determine the basis of obligate biotrophism of AMF (see Bago and Bécard, 2002). To do this, early modifications of the monoxenic culture system were designed: roots were physically separated from the monoxenic culture either by separating root and fungus with a dialysis membrane (Bécard and Piché 1989a), or by using different compartments in which both symbionts would develop with no physical contact (Bécard and Piché 1989b). These studies allowed to pinpoint CO_2 as a major signal factor in the host–fungus recognition process. From then on, monoxenic systems have been modified and redesigned to address fundamental questions on the biology of the symbiosis.

A major step which greatly pushed forward mycorrhizal research was St-Arnaud et al.'s (1996) two-compartment monoxenic system in which extraradical, root-free (yet symbiotic) fungal mycelium could be obtained. On publication, it was immediately adopted by different laboratories around the world. Knowledge on the metabolism of AMF has greatly benefited from the use of compartmentalized monoxenic cultures, since researchers have now the possibility to precisely control the media composition for extraradical mycelium growth. Combined with different types of in vitro culture systems this can offer many different experimental possibilities. It could be used, for instance, with liquid medium added into the hyphal compartment to conduct pulse-chase experiments (Fig. 4a), as carried out by Maldonado-Mendoza et al. (2001) to isolate a first P transporter in AMF extraradical mycelium. If used with solid M-C medium, labeling of the hyphal compartment could be conducted by mixing directly to the culture medium either prior to (in the

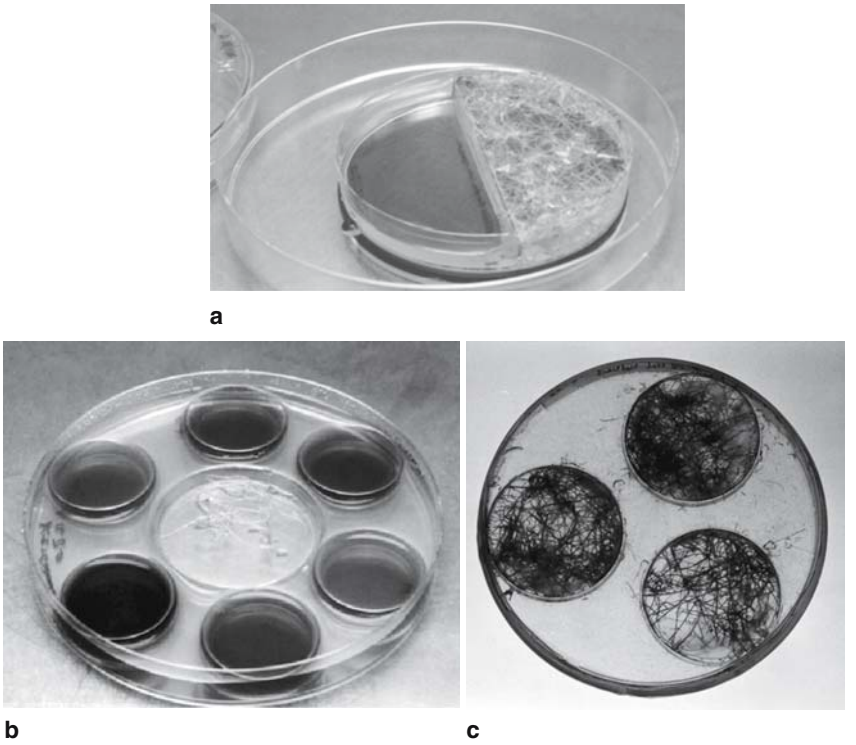

Fig. 4 Multicompartmented systems used for in vitro AM cultures. **a** St-Arnaud's bicompartmented Petri plate with liquid medium on the hyphal compartment. Note the developing ERM on it. **b** Multicompartmented 150-mm Petri plates with a single monoxenic central culture to test fungal colony morphogenesis on different nutrient availability. **c** Multicompartmented 150-mm Petri plates with three different monoxenic cultures to test fungal colony competition between extraradical hyphae

case of term stable compounds) or after (compounds that will require filter-sterilizing) autoclave sterilization. Labeling could also be by amending the solidified medium with the compound to test, either by adding dropwise or into a labeling well.

By using this approach, it is now known that AMF are able to utilize either inorganic (Bago et al. 1996; Villegas et al. 1996; Toussaint et al. 2004) or organic (Capellazzo et al. 2007) nitrogen, and translocation and utilization of this key element is better understood (Bago et al. 2001; Govindarajulu et al. 2005; Jin et al. 2005). Furthermore, a high affinity ammonium transporter has been isolated and characterized by altering the nitrogen levels and the nitrogen source in the fungal compartment (López-Pedrosa et al. 2006). Combining monoxenic cultures with ^{32}P and image analyses, Joner et al. (2000) were able to visualize and localize P uptake by ERM and to evaluate the rate of transfer of P within the mycelium. Organic P hydrolysis by ERM has also been assessed by using bicompartmented monoxenic cultures (Koide and Kabir, 2000).

Studies by Bago et al. (1999b, 2000, 2002, 2003), Pfeffer et al. (1999, 2001) and Lammers et al. (2001) have combined ^{13}C-labelling with monoxenic cultures, and retraced it by NMR to follow the destination and the chemical transformations of incorporated C. It was concluded that triacylglycerides are the most important C storage compound in AMF and that the ERM and spores depend entirely on lipid synthesis by intraradical mycelium, and subsequent transport to the extraradical colony to ensure nutrient availability to the rest of the colony.

The media of monoxenic cultures could also be amended for the study of the mechanisms of adaptation by AMF to environmental stresses, such as heavy metals or saline conditions. For instance, Pawlowska and Charvat (2004) supplemented the fungal compartment of *G. intraradices* and *G. etunicatum* monoxenic cultures with Zn^{2+}, Cd^{2+} or Pb^{2+} and studied the development of the fungal colony. In this study, they were able to conclude that the sporulation was more sensitive to these heavy metals than the expansion of the colony, and suggested that AMF would sacrifice sporulation in order to reach more favorable environments. RNA has been extracted from the extraradical mycelium grown in the presence of heavy metals to make subtractive suppressive hybridization (SSH) libraries (Ouziad et al. 2004; Waschke et al. 2006) in order to identify genes involved in metal homeostasis, such as glutathione-S-transferases (Waschke et al. 2006), or a putative Zn^{2+}-transporter of the ZIP family of metal transporters (Hildebrandt et al. 2006). Expressed-sequence tags (ESTs) obtained from monoxenic cultures have also provided sequences to clone a Zn^{2+}-transporter of the CDF family (González-Guerrero et al. 2005). An oxidative-stress responsive metallothionein (González-Guerrero et al. 2007) has also been characterized by this approach. In addition, monoxenic cultures can also be used to study the effect of salt stress on fungal development (Jahromi et al. 2007) or to characterize the expression levels of saline-responsive genes (Porcel et al. 2006).

All the research described above highlights the versatility of monoxenic cultures to study AMF biology at a biochemical level (metabolism of N, P and C), at a morphological level (fungal development under stress) or at a molecular level (regulation

of gene expression). Yet there is still much more that can be achieved. DNA obtained from monoxenic cultures is being used in the current *G. intraradices* genome sequencing project (*http://darwin.nmsu.edu/~fungi/index.php*). Furthermore, monoxenic cultures can be used for protein extraction, and therefore to study the proteome. At later stages, by linking gene expression and proteins to higher level of biological functions, the molecular fluxes through metabolic networks (the fluxome) can be determined.

However, there are some aspects of monoxenic cultures that need to be addressed and resolved in order to greatly expand the flexibility of this tool. As mentioned above, increasing the number of available AMF isolates to generalize the research results previously obtained with *G. intraradices* or *Gigaspora margarita* results mandatory; in this regard, the comparison of tolerant versus non-tolerant ecotypes, once microarrays become available, will result in a greater understanding of the adaptability of AMF. Finally, and taking into account that biological events occurring within the AMF colony are transient and localized, new techniques should be developed/improved (e.g., in situ PCR) to comply with such request in order to fully understand the bases of this fascinating yet elusive symbiosis.

2.3.2 Multicompartmented Systems to Study Fungal Colony Development and Interactions

Four years ago, a new modification of the monoxenic culture system was designed: the multicompartment in vitro system (Fig. 4b, c). In a first study (Bago et al. 2004), the system consisted of a large Petri plate (150 mm diameter) in which a number of smaller Petri plates were arranged, each one of these containing a different nutrient-amended culture medium. A medium-sized Petri plate was placed in the centre of the large plate containing an AM monoxenic culture (the 'culture compartment'). The space in between the plates was filled by a nutrient-free culture medium. Throughout the experiment, ERM from the monoxenic culture core, but not roots, were allowed to exit the culture compartment and develop onto the different external media of the system. This study allowed the observation of striking morphogenetic changes in fungal development and colony architecture due to the effect of nutrients in the culture medium, and highlighted the enormous plasticity and adaptability of the fungus to its environment.

More recently, a slight modification of the multi-compartment in vitro system has been presented (Cano and Bago 2005) and in this case, medium-sized Petri plates each containing a different monoxenic culture were arranged inside a large Petri plate (Fig. 4c). The space between the inner plates was filled with a low nutrient culture medium, and ERM of the different fungal colonies (but not roots) were allowed to develop in this intermediate area, thereby free to interact and compete for existing nutrients. Hyphal interactions were followed in situ and possible competition and strategies for substrate colonization between the different AMF studied. The results demonstrated that little or no competition occurred between the ERM of different AMF; on the contrary, they seemed to 'sense' each other and

avoid occupying the same zone of the substrate. This avoidance has tremendous ecological significance and may help us to understand the complicated relations between different microorganisms in nature.

2.4 Methods for Quantifying Mycorrhizal Development in vitro

Up to now we have summarized the different uses of AM in the in vitro culture system for studying mycorrhizal biology and interactions. These studies in most cases require the measurement of root and fungal development within the culture medium. We will now summarize the three currently used methods to carry out such quantifications.

2.4.1 Recovery-and-Weight Destructive Method

This method consists of liquefying the culture medium of the plate or culture compartment we want to quantify, recovering the mycorrhizal material in it by sieving or filtration and then weighing. To achieve this, the medium is separated from the compartment using a scalpel and transferred to the container where liquefaction of the medium takes place. The liquefaction procedure will depend on the agar-like agent used in the culture medium: if this is a agar–agar (e.g., Bacto-Agar, Bago et al. 1996) it liquefies just by heating at 90 °C. However, if the medium contains a jellifying agent such as PhytaGel® or GelGro®, it will not dissolve by heat and, in this case, the sodium citrate technique (Doner and Bécard 1991), in which the culture medium is mixed with a 10-mM solution of cold sodium citrate and maintained in suspension for 5 min, would be used. This method is easy, quick and is the one of preference for obtaining material for molecular biology or biochemical studies (as the material can be immediately frozen at −80 °C without heat damage). However, it is a destructive method which will not give us any information about the developmental/morphogenetic state of the fungal colony at the time of collection.

2.4.2 Gridline Intersect 3D Method

The Gridline intersect 3D method (Bago et al. 1998a) can be used to quantify mycorrhizal development at the end of an experiment. It is in fact an adaptation of the traditional gridline intersect method used for estimating mycorrhizal colonization in roots (Tennant 1975) and is modified for the monoxenic culture in vivo conditions. Briefly, several 1 × 1 cm grid squares are placed randomly on the bottom of the Petri plate. The observer counts the number of either root or hyphal intersections crossing the lines of the grid and calculates the results as root/extraradical runner hyphal length according to the following formula:

Total length root/runner hyphae per cm^2 = 11/14 × number of intersection counts × grid size (i.e., 1 cm^2).

With this calculation, the length of root and extraradical runner hyphae are obtained. To complete the quantification, the number of BAS and spores using the same selected squares should also be counted. Both root or hyphal length and BAS or spore counts are performed three-dimensionally, i.e. by exploring the full depth of the culture medium at each screened spot. To make quantification easier, fungal tissue in situ can be stained before counting (trypan blue stain recommended; see Section 2.2). Combining all these data, substrate colonization maps of the Petri plates can be produced (Cano and Bago 2005). This also allows indicative morphogenetic ratios (such as runner hyphal length / root length, BAS / runner hyphae, and spore / runner hyphae) to be calculated. Although this method is quite complicated, tiring and time-consuming, it remains the most appropriate to evaluate in vitro mycorrhizal development, especially for fungal colony growth and the formation of BAS and spores.

2.4.3 Estimation-by-asterisk in vivo Method

As mentioned, the above-described gridline 3D quantification method is quite time-consuming, which makes it almost impossible to use if we want continuous in vivo tracking of the development of the AM fungal colony. Therefore, the estimation-by-asterisk method is recommended (Bago et al. 2004; Dickson et al., unpublished). This method consists of estimating the colony spread on a subjective scale basis ranging from one asterisk (approximately 20% of the culture medium containing mycelium, or a spore density of 200 spores/cm^2) to five asterisks (100% of medium containing mycelium, or a spore density of approximately 1,000 spore/cm^2). A stereomicroscope at low magnification is used for colony assessment. The developmental stage of the colony should also be mentioned and can be indicated as the following: Abs, absorptive phase (i.e., presence of runner hyphae and BAS with almost no spores); Abs/Sp, transition between absorptive to sporulative phases; Sp/Abs, colony predominantly in sporulative phase; and Sp, terminal sporulative phase (Cano and Bago 2005).

In a complete study, the three above-mentioned techniques can be used sequentially: day by day in situ following of the colony uses the estimation-by-asterisks procedure, global quantification at the end of the experiment is carried out by the gridline intersect 3D method, and in cases where molecular analyses will be performed, the recovery-and-weigh method can be implemented.

3 Three New in vitro Techniques That Will Make AM Research Easier

The final part of this chapter will be devoted to describe three new in vitro techniques developed in our laboratory.

3.1 Rapid in vitro Colonization of Roots ("RapInCoR"): The Easiest and Fastest Way to Obtain High-Quality Mycorrhizas

One of the major drawbacks researchers find when using in vitro cultures of AM is the low percentage of colonized root tissue obtained (usually <20%) and most importantly, the long period of time (ca. 8 weeks) needed to reach this. This slow development of colonization means that the roots are old and not very well suited for molecular biology analyses to be conducted with them.

In an attempt to alleviate this problem an experimental system has been designed to quickly obtain freshly-colonized mycorrhizal root tissue in vitro (Fig. 5). This 'RapInCoR' method consists of using a traditional two-compartment AM monoxenic culture. One week after the ERM has passed over the plastic barrier of the plate into the hyphal compartment (HC) medium (which should be full

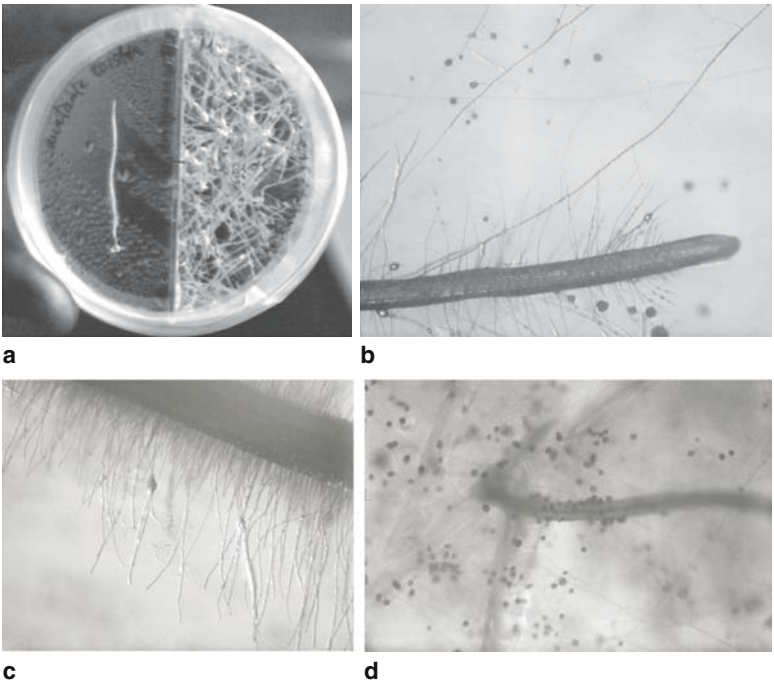

Fig. 5 The rapid in vitro colonization of roots (RapInCoR) experimental system to obtain quick and clean AM material for biochemical and/or molecular biology determinations. **a** A general view of the system. **b** Fungal extraradical hyphae approaching to the newly-deposited host root. **c** Extraradical hyphae contacting root hairs and developing appressoria on the newly-deposited root. **d** As the newly-deposited root develops, existing ERM 'embraces' it, thus maximizing possibilities of becoming quickly colonized

'M', i.e., with sucrose) the ERM advances quite quickly and is in an almost-pure absorptive phase. This will take approximately 6–8 weeks from set-up. At that time, a young vigorously-developing root is placed onto the HC medium, in the close vicinity of the fungal ERM growing front (Fig. 5a). The AM runner hyphae quickly approach the young root (Fig. 5b), interacts with it (Fig. 5c), and envelops it thoroughly (Fig. 5d). After 1 week, colonization tests revealed a mean colonization of 25% in the young root. This colonization percentage does not significantly increase even if the root is left on culture for another week. Therefore, in effect, the RapInCoR method allows maximum AM in vitro colonization to be reached very quickly and the very young actively-growing mycorrhizal root appears to be exceptionally well-suited for testing gene expression (Cano et al., unpublished). The future of this new technology will be to combine it with the in situ localization of intraradical AM structures using in vivo microscopy; this would allow us to select only the young root's colonized areas, thus minimizing the above-mentioned dilution effect and producing unique experimental material for gene expression studies.

3.2 The "Cut'n-go" Technique for Synchronizing Growth of ERM

A second frequent problem encountered by researchers when using in vitro AM cultures for basic research is the fact that, although clonal, AM fungal development in replicate plates within a given experiment do not usually grow in a synchronic way. For example, in an experiment of 10 replicates, after 8 weeks of growth you may have two plates in sporulative phase, six in absorptive-to-sporulative phase and two in initial absorptive phase. Unfortunately, this is frequently not considered by researchers. This is a major drawback especially when performing molecular biology tests as inconsistent results may come from the differential expression of genes from tissue used at various developmental stages (Bago and Cano 2005). An easy way to avoid this is what we call the 'cut'n-go method'. Experiments are conducted in two-compartment Petri plates as before. When all the replicate plates within an experiment have ERM which has passed over the plastic barrier and been growing for at least 1 week in the M-C medium, the entire M-C medium is cut out and removed from the plate. Immediately after removal, warm fresh M-C medium is added to the emptied HC, left to solidify and plates are then put back to incubate. The cutting of the ERM invigorates it, and new mycelium develops as a compact colony front to cross the barrier and colonize the fresh culture medium. This procedure allows synchronized growth of ERM in the different replicate plates, thus making it possible to get fungal tissue at similar developmental stages (and most probably with similar gene expression pattern).

3.3 Whole Plant In Vitro Cultures: A Step Forward to Understanding Mycorrhizas

As previously mentioned, one of the most common criticisms made to the AM monoxenic system using ROCs as hosts is that this is in fact an artificial mycorrhiza, since there is no shoot and therefore the root is fed unnaturally via the culture medium. Although valid for studying AMF biology, as well as some aspects of root/fungal interactions (Bago et al. 2006; Dickson et al., unpublished), the absence of a functional whole plant is an important aspect researchers must take into account when using monoxenic cultures with ROCs. For instance, studying the influence of the shoot on the AM fungus is simply not possible under these conditions, nor is the study of systemic responses induced in the plant by mycorrhization, nor tracking the fate of nutrients from root to shoot and vice versa. The recent development of an in vitro whole plant mycorrhizal system (Voets et al. 2005; Dupré de Boulois et al. 2006) using as a basis the monoxenic one- or two-compartment system with solid culture media, will offer, no doubt, major advances in mycorrhizal research. We present here an additional method which mostly addresses the need of basic research for getting large amounts of mycorrhizal tissue from a whole-plant in vitro culture which would be well colonized, easy to label, easy to recover and which would allow sequential harvesting.

The system consists of a Kitasato beaker filled with liquid 'M' medium, in whose top a nylon mesh bag, containing a portion of a former AM monoxenic culture is introduced (Figs. 6a–e). On the top of this, a surface-sterilized seedling is placed. The bag should lightly touch the liquid medium so that both fungal inoculum and seed are kept moist (Fig. 6b). The system is covered by a plastic sunbag and through the lateral opening of the Kitasato's flask, sterile air is gently pumped so that the liquid medium is kept well oxygenated (Fig. 6c). This experimental system allows synchronous in vitro development of both AM fungal infective mycelium and seedling (Fig. 6b) which readily gets colonized (20% colonization after 4 weeks). AM colonization is preferentially located at zones close to the nylon mesh bag. Moreover, this system allows the continuous production of mycorrhizal roots as it can be opened under sterile conditions, roots cut and collected, and the bag containing the inoculated plant put back to the system for mycorrhizas to regrow (Fig. 6d). As mentioned above, such system should be linked to in situ detection of AM colonization to avoid dilution.

This experimental design has been tested with different plants (Fig. 6e) and AM fungi and given excellent results. The perfect model plant, however, would of course be *Arabidopsis thaliana* whose plant genome has been fully sequenced. Unfortunately, this plant belongs to the largely nonmycorrhizal family Brassicaceae. The thought of screening many hundreds (or thousands perhaps) of generated mutants is daunting, but if a mycorrhizal plant could be discovered then it would be a worthwhile exercise with possibilities of determining the genes that 'encourage' colonization.

A modification of this whole plant in vitro system has been used to 'tame' AM fungi (such as *G. mosseae*) which has been reluctant to develop in the in vitro conditions, (Figs. 6f–i). This consists of putting pot culture mycorrhizal inoculum instead of monoxenic material into the nylon mesh bag and placing a surface-sterilized seedling on top of it (Fig. 6f). After 4 weeks, colonized roots (Figs. 6g, h) with intraradical fungal structures (reaching up to 25% colonization in certain zones, Fig. 6i) were obtained. This method, besides of being of interest to obtain semi-pure, controlled mycorrhizal tissue opens new possibilities for increasing the

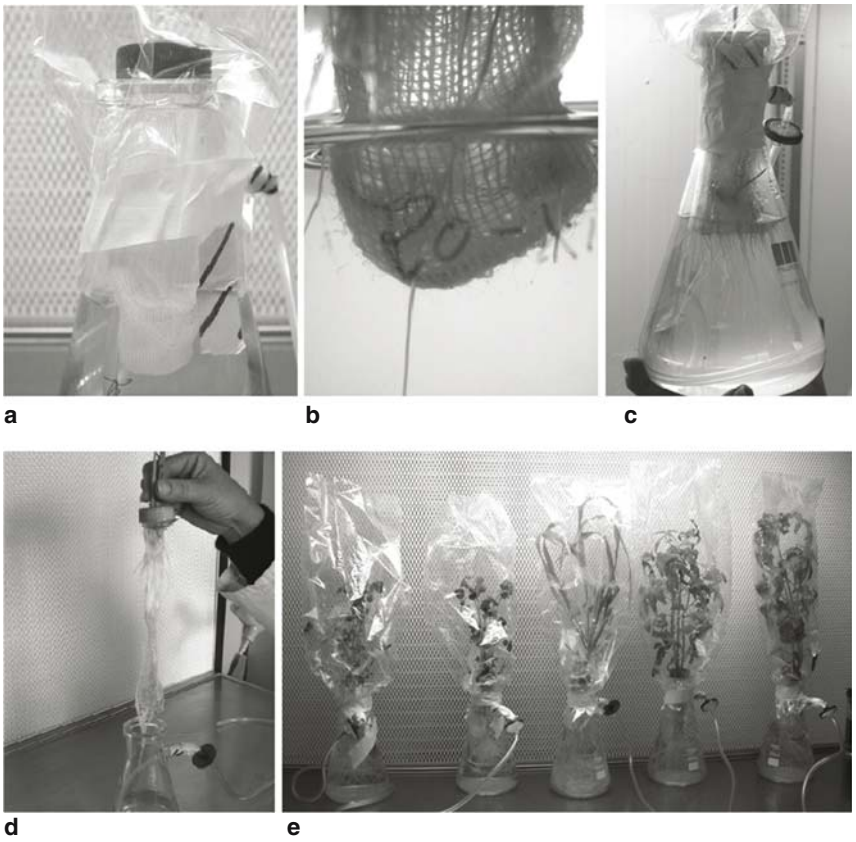

Fig. 6 Whole-plant in vitro AM cultures in liquid culture media: **a–e** From a previous monoxenic culture; **e–i** from an open-pot pure AM inoculum. **a,f** Overview of the experimental setup. **b** Roots entering the liquid medium after passing through the monoxenic inoculum cube. **c,g** Overview of the system when roots have extensively developed in the liquid medium. **d** Extraction of the colonized roots under sterile conditions. **e** Overview of the experimental setup with the different plant tested (tomato, clover, sorghum). **h** Extraradical mycelium develops close to the root in the liquid medium (*arrow*). **i** Roots colonized by *G. mosseae* using this experimental design

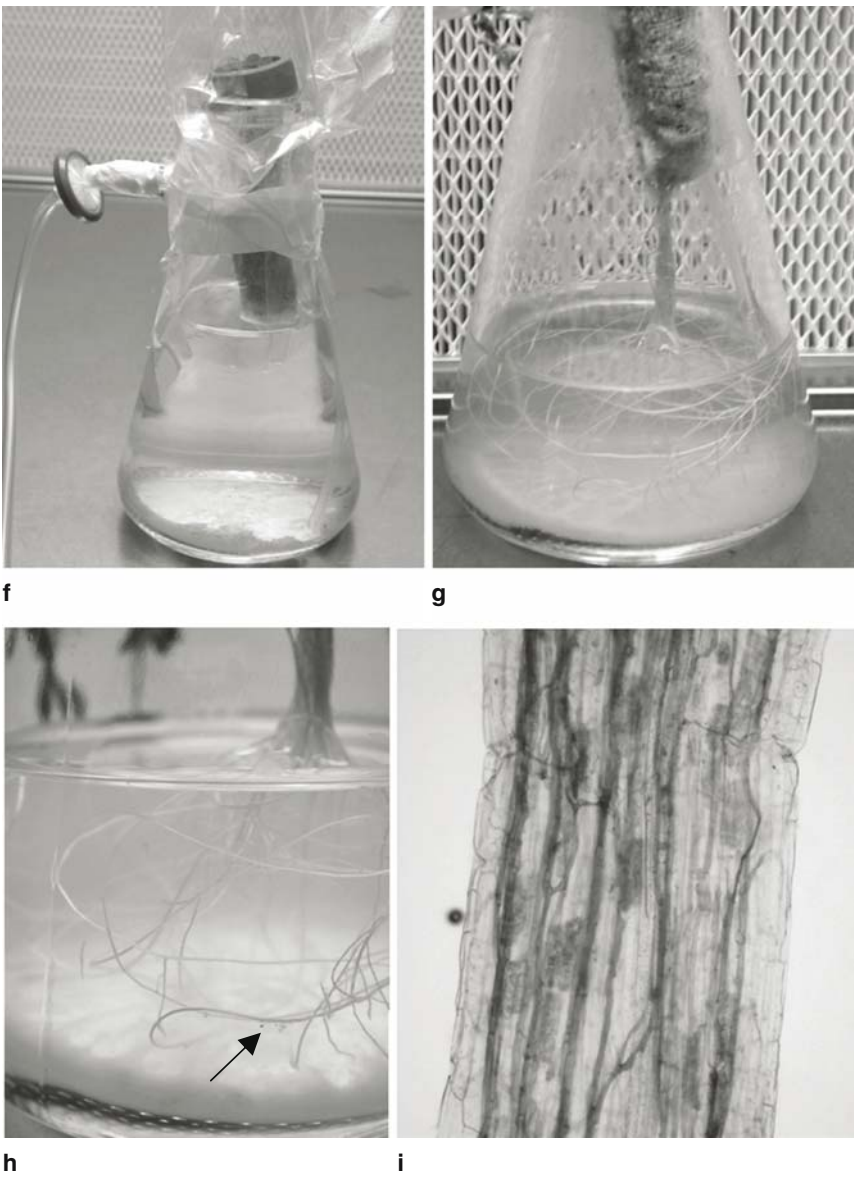

Fig. 6 (continued)

number of AM fungal isolates cultured in vitro. Of course, more research is needed to polish these techniques and to make them easy for general use. Nevertheless, the basis of the 'AM in vitro era' is already settled.

4 Conclusions

In vitro culture systems offer enormous versatility and potential to be accommodated and applied in many fields of arbuscular mycorrhiza basic research. Exponentially-increased knowledge on the intrinsic bases of the symbiosis has been obtained during the less than 10 years since monoxenic cultures were widely used; yet the best is still to come. In the near future, the coupling of powerful emerging research fields such as genomics, proteomics, metabolomics and fluxomics with cutting-edge technologies (in vivo microscopy, in situ molecular biology, four-dimensional determinations), with the increasing number of AMF isolates available in vitro, and with the unique and necessary inventive force of mycorrhizologists, will allow us to translate basic research it into a respectful, integrated and wide use of arbuscular mycorrhiza, as our society demands.

References

Bago B (1998) AM monoxenic cultures using tomato non-transformed roots. In: Kling M (ed) Development and function of the mycelium of arbuscular mycorrhizal fungi. Dept. Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, pp 41–44

Bago B, Bécard G (2002) Bases of the obligate biotrophy of arbuscular mycorrhizal fungi. In: Mycorrhiza technology: from genes to bioproducts –achievements and hurdles in arbuscular mycorrhizal research. Birkhäuser, Basel, pp 33–48

Bago B, Cano C (2005) Breaking myths on arbuscular mycorrhizas in vitro biology. In: Declerck S, Strullu DG, Fortin A (eds) In vitro biology of mycorrhizal symbiosis. Soil Biology Series, vol 4. Springer, Heidelberg, pp 111–138

Bago B, Vierheilig H, Piché Y, Azcón-Aguilar C (1996) Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular-mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Bago B, Azcón-Aguilar C, Piché Y (1998a) Architecture and developmental dynamics of the external mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown under monoxenic conditions. Mycologia 90:52–62

Bago B, Zipfel W, Williams RC, Chamberland H, Lafontaine J-G, Webb WW, Piché Y (1998b) In vivo studies on the nuclear behavior of the arbuscular mycorrhizal fungus *Gigaspora rosea* grown under axenic conditions. Protoplasma 203:1–15

Bago B, Zipfel W, Williams RM, Piche Y (1999a). Nuclei of symbiotic arbuscular mycorrhizal fungi as revealed by in vivo two-photon microscopy. Protoplasma 209:77–89

Bago B, Pfeffer PE, Douds DD Jr., Brouillette J, Bécard G, Shachar-Hill Y (1999b) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–271

Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 124:949–958

Bago B, Pfeffer P, Shachar-Hill Y (2001) Could the urea cycle be translocating nitrogen in the arbuscular mycorrhizal symbiosis? New Phytol 149:4–8

Bago B, Zipfel W, Williams RM, Jun J, Arreola R, Lammers PJ, Pfeffer PE, Shachar-Hill Y (2002) Translocation and utilization of fungal storage lipid in the arbuscular mycorrhizal symbiosis. Plant Physiol 128:108–124

Bago B, Pfeffer PE, Abubaker J, Jun J, Allen JW, Brouillette J, Douds DD, Lammers PJ, Shachar-Hill Y (2003) Carbon export from arbuscular mycorrhizal roots involves the translocation of carbohydrate as well as lipid. Plant Physiol 131:1496–507

Bago B, Cano C, Azcón-Aguilar C, Samson J, Coughlan AP, Piché Y (2004) Differential morphogenesis of the extraradical mycelium of an arbuscular mycorrhizal fungus grown monoxenically on spatially heterogeneous culture media. Mycologia 96:452.462

Bago B, Cano C, Toussaint J-P, Smith S, Dickson S (2006) Interactions between the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* and non-transformed tomato roots of either wild-type or AM-defective phenotypes in monoxenic cultures. Mycorrhiza 16:429–436

Balaji B, Ba AM, LaRue TA, Tepfer D, Piché Y (1994) *Pisum sativum* mutants insensitive to nodulation are also insensitive to invasion in vitro by the mycorrhizal fungus *Gigaspora margarita* Plant Sci 102:195–203

Barker SJ, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbioses. J Plant Growth Regul 19:144–154

Bécard G, Fortin A (1988) Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol 108:211–218

Bécard G, Piché Y (1989a) Fungal growth stimulation by CO_2 and root exudates in vesicular-arbuscular mycorrhizal symbiosis Appl Environ Microbiol 55:2320–2325

Bécard G, Piché Y (1989b) New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita* New Phytol 112:77–83

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker DG (2001) Agrobacterium rhizogenes-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol Plant Microbe Interact 14:695–700

Brundrett MC, Piché Y, Peterson RL (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can J Bot 62:2128–2134

Cano C, Bago A (2005) Competition and substrate colonization strategies of three polyxenically-grown arbuscular mycorrhizal fungi. Mycologia 97:1201–1214

Cappellazzo G, Lanfranco L, Bonfante PA (2007). Limiting source of organic nitrogen induces specific transcriptional responses in the extraradical structures of the endomycorrhizal fungus *Glomus intraradices*. Curr Genet 51:59–70

Cavagnaro TR, Gao L-L, Smith FA, Smith SE (2001) Morphology of arbuscular mycorrhizas is influenced by fungal identity. New Phytol 151:469–475

Chabot S, Bel-Rhlid R, Chênevert R, Piché Y (1992a) Hyphal growth promotion in vitro of the VA mycorrhizal fungus, *Gigaspora margarita* Becker & Hall, by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. New Phytol 122:461–467

Chabot S, Bécard G, Piché Y (1992b) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Cranenbrouck S, Voets L, Bivort C, Renard L, Strullu DG, Declerck, S (2005) Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck S, Strullu DG, Fortin A (eds) In vitro biology of mycorrhizal symbiosis. Soil biology series, vol 4. Springer, Heidelberg, pp 341–375

Declerck S, Strullu DG, Fortin A (eds) (2005) In vitro culture of mycorrhizas. Soil biology series, vol 4. Springer, Heidelberg

Dickson S (2004) The *Arum-Paris* continuum of mycorrhizal symbioses. New Phytol 163:187–200

Dickson S, Kolesik P (1999) Visualisation of mycorrhizal fungal structures and quantification of their surface area and volume using laser scanning confocal microscopy. Mycorrhiza 9:205–213.

Dickson S, Schweiger PF, Smith FA, Söderström B, Smith S (2003) Paired arbuscules in the *Arum*-type arbuscular mycorrhizal symbiosis with *Linum usitatissimum* L. Can J Bot 81:457–463

Dickson S, Smith FA, Smith SE (2007) Structural differences in arbuscular mycorrhizal symbioses: more than 100 years after Gallaud, where next? Mycorrhiza, online DOI 10.1007/s00572–007–0130–9

Doner LW, Bécard G (1991) Solubilization of gellan gels by chelation of cations. Biotechnol Technol 5:25–28

Douds DD (1997) A procedure for the establishment of *Glomus mosseae* in dual culture with Ri T-DNA-transformed carrot roots. Mycorrhiza 7:57–61

Dupré de Boulois H, Voets L, Delvaux B, Jakobsen I, Declerck S (2006) Transport of radiocaesium by arbuscular mycorrhizal fungi to *Medicago truncatula* under in vitro conditions. Environ Microbiol 8:1926–1934

Fortin JA, Bécard G, Declerck S, Dalpe Y, St-Arnaud M, Coughlan AP, Piche Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20

Gallaud I (1904) Études sur les mycorrhizes endotrophes. Rev Génet Bot, Bigot Frerès, Lille

Gallaud I (1905) Études sur les mycorrhizes endotrophes. Rev Gén Bot 17:5–48, 66–83, 123–135, 223–239, 313–325, 425–433, 479–500

Gao, L-L (2002) Control of arbuscular mycorrhizal colonisation: studies of a mycorrhiza-defective tomato mutant. PhD thesis, The University of Adelaide, Australia

Gao L-L, Knogge W, Delp G, Smith FA, Smith SE (2004) Expression patterns of defense-related genes in different types of arbuscular mycorrhizal development in wild-type and mycorrhiza-defective mutant tomato. Mol Plant Microbe Interact 17:1103–1113

Gavito ME, Olsson PA, Rouhier H, Medina-Penafiel A, Jakobsen I, Bago A, Azcón-Aguilar C (2005) Temperature constraints on the growth and functioning of root organ cultures with arbuscular mycorrhizal fungi. New Phytol 168:179–188

Giovannetti M, Fortuna, P, Citernesi, AS, Morini, S, Nuti, MP (2001) The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. New Phytol 151:717–724

Glassop D, Smith SE, Smith FW (2005) Cereal phosphate transporters associated with the mycorrhizal pathway of phosphate uptake into roots. Planta 222:688–698

González-Guerrero M, Azcón-Aguilar C, Mooney M, Valderas A, MacDiarmid CW, Eide DJ, Ferrol N (2005) Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Fungal Genet Biol 42:130–40

González-Guerrero M, Cano C, Azcón-Aguilar C, Ferrol N (2007).*GintMT1* encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress. Mycorrhiza 17:327–335

Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–23

Grace C, Stribley DP (1991) A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. Mycol Res 95:1160–1162

Hepper CM (1984) Isolation and culture of VA mycorrhizal (VAM) fungi. In: Powell CL, Bagjaraj DJ (eds) VA mycorrhizae, CRC Press, pp 95–112

Hildebrandt U, Regvar M, Bothe H (2006) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146

Jahromi F, Aroca R, Porcel R, Ruiz-Lozano JM (2007) Influence of salinity on the *in vitro* development of *Glomus intraradices* and on the in vivo physiological and molecular responses of mycorrhizal lettuce plants. Microb Ecol (in press)

Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y (2005) The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol 168:687–696

Joner EJ, Ravnskov S, Jakobsen I (2000) Arbuscular mycorrhizal phosphate transport under monoxenic conditions using radio-labelled inorganic and organic phosphate. Biotechnol Lett 22:1705–1708

Juge C, Samson J, Bastien C, Vierheilig H, Coughlan A, Piché Y (2002) Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period. Mycorrhiza 12:37–42

Karandashov V, Kuzovkina I, Hawkins HJ, George E (2000) Growth and sporulation of the arbuscular mycorrhizal fungus *Glomus caledonium* in dual culture with transformed carrot roots. Mycorrhiza 10:23–28

Karandashov V, Nagy R, Wegmüller S, Amrhein N, Bucher M (2004) Evolutionary conservation of a phosphate transporter in the arbuscular mycorrhizal symbiosis. Proc Natl Acad Sci USA 101:6285–6290

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus *Glomus intraradices* can hydrolyse organic phosphate. New Phytol 148:511–517

Kormanik PP, McGraw AC (1982) Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Schenck NC (ed) Methods and principles of mycorrhizal research. The American Phytopathological Society, St Paul, Minn., pp 37–45

Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 92:486–505

Lammers PJ, Jun J, Abubaker J, Arreola R, Gopalan A, Bago B, Hernandez-Sebastia C, Allen JW, Douds DD, Pfeffer PE, Shachar-Hill Y (2001) The glyoxylate cycle in an arbuscular mycorrhizal fungus. Carbon flux and gene expression. Plant Physiol 127:1287–1298

Lopez-Pedrosa A, González-Guerrero M, Valderas A, Azcón-Aguilar C, Ferrol N (2006) *GintAMT1* encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of *Glomus intraradices*. Fungal Genet Biol 43:102–110

Maldonado-Mendoza IE, Dewbre GR, Harrison MJ (2001) A phosphate transporter gene from the extraradical mycelium of an arbuscular mycorrhizal fungus *Glomus intraradices* is regulated in response to phosphate in the environment. Mol Plant Microbe Interact 14:1140–1148

Mosse B (1962) The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. J Gen Microbiol 27:509–520

Mosse B, Hepper C (1975) Vesicular-arbuscular mycorrhizal infections in root organ cultures. Physiol Plant Pathol 5:215–223

Mugnier J, Mosse B (1987) Vesicular-arbuscular mycorrhizal infections in transformed root-inducing T-DNA roots grown axenically. Phytopathology 77:1045–1050

Müller J, Mohr U, Sprenger N, Bortlik K, Boller T, Wiemken A (1999) Pool sizes of fructans in roots and leaves of mycorrhizal and non-mycorrhizal barley. New Phytol 142:551–559

Ouziad F, Hildebrandt U, Schmelzer E, Bothe H (2005) Differential gene expressions in arbuscular mycorrhizal-colonized tomato grown under heavy metal stress. J Plant Physiol 162:634–649

Pawlowska TE, Charvat I (2004) Heavy-metal stress and developmental patterns of arbuscular mycorrhizal fungi. Appl Environ Microbiol 70:6643–6649

Pawlowska TE, Douds DD, Charvat I (1999) In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycol Res 103:1549–1556

Pfeffer PE, Douds DD, Bécard G, Shachar-Hill Y (1999) Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. Plant Physiol 120:587–598

Pfeffer PE, Bago B, Shachar-Hill Y (2001) Exploring mycorrhizal function with NMR spectroscopy. New Phytol 150:543–553

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Plenchette C, Declerck S., Diop TA, Strullu GD (1996) Infectivity of monoaxenic subcultures of the arbuscular mycorrhizal fungus *Glomus versiforme* associated with Ri-T-DNA-transformed carrot root. Appl Microbiol Biotechnol 46:545–548

Porcel R, Aroca R, Cano C, Bago A, Ruíz-Lozano JM (2006) Identification of a gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding for a 14–3-3 protein that is up-regulated by drought stress during the AM symbiosis. Microbiol Ecol 52:575–582

Sanders, IR (2004) Intraspecific genetic variation in arbuscular mycorrhizal fungi and its consequences for molecular biology, ecology, and development of inoculum. Can J Bot 82:1057–1062

Smith FA, Smith SE (1997) Structural diversity in (vesicular)-arbuscular mycorrhizal symbiosis. New Phytol 137:373–388

Smith SE, Smith FA, Jakobsen I (2004) Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol 162:511–524

St-Arnaud M, Elsen A (2005) Interaction of arbuscular mycorrhizal fungi with soil-borne pathogens and non-pathogenic rhizosphere micro-organisms. In: Declerck S, Strullu DG, Fortin A (eds) *In vitro* biology of mycorrhizal symbiosis. Soil biology series, vol 4. Springer, Heidelberg, pp 216–231

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *G. intraradices* in an *in vitro* system in the absence of host roots. Mycol Res 100:328–332

Strullu DG, Romand C (1986) Méthode d'obtention d'endomycorhizes á vésicules et arbuscules en conditions axéniques. Physiol Végét 303:245–250

Tennant D (1975) A test of a modified line intersect method of estimating root length. J Ecol 63:995–1001.

Toussaint JP, St-Arnaud M, Charest C. 2004. Nitrogen transfer and assimilation between the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith and Ri T-DNA roots of *Daucus carota* L. in an in vitro compartmented system. Can J Microbiol 50:251–260

Vierheilig H, Bago A (2005) In: Declerck S, Strullu DG, Fortin A (eds) *In vitro* biology of mycorrhizal symbiosis. Soil biology series, vol 4. Springer, Heidelberg, pp 139–158

Vierheilig H, Coughlan AP, Wyss U, Piche Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64:5004–5007

Villegas JR, William RD, Nantais L, Archambault J, Fortin JA (1996) Effect of N source on pH and nutrient exchange of extrametrical mycelium in a Ri T-DNA transformed root system. Mycorrhiza 6:247–251

Voets L, Dupré de Boulois H., Renard L, Strullu DG, Declerck S (2005) Development of an autotrophic culture system for the in vitro mycorrhization of potato plantlets. FEMS Microbiol Lett 248:111–118

Waschke A, Sieh D, Tamasloukht M, Fischer K, Mann P, Franken P (2006) Identification of heavy metal-induced genes encoding glutathione S-transferases in the arbuscular mycorrhizal fungus *Glomus intraradices*. Mycorrhiza 17:1–10

White NS, Errington RJ, Fricker MD, Wood JL (1996) Aberration control in quantitative imaging of botanical specimens by multidimensional fluorescence microscopy. J Microsc 181:99–116

White PR (1934) Tomato root organ culture. Plant Physiol 9:585–600

Interactions of *Piriformospora indica* with Medicinal Plants

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1 Introduction

The microbial world exerts a negative as well a positive impact on living plants and animals, and forms an association either pathogenic or symbiotic with the other partners of the living world. Mycorrhiza refers to an association or symbiosis between plants and fungi that colonize the roots during periods of active plant growth. The intimate symbiotic relationships developed between mycorrhizal fungi and plants, since the colonization of land by the latter, have led to interdependence between these organisms for many basic processes. The fungi require plants to accomplish their life cycle. Plants depend heavily on mycorrhizal fungi for many different functions, such as mineral nutrition and abiotic and biotic stress resistance. Substantial evidence has accumulated in the recent past about how the use of the microsymbiont could significantly contribute in decreasing use of fertilizers and pesticides in agriculture, forestry and flori-hortriculture, especially if combined with other beneficial soil microorganisms.

The most common and prevalent arbuscular mycorrhizal fungi play an indispensable role in upgrading plant growth, vigor and survival by a positive impact on the nutritional and hydratic status of the plant and on soil health, by increasing the reproductive potential, improving root performance, and providing a natural defence against invaders, including pests and pathogens. The described species of arbuscular mycorrhizal fungi mainly belong to Zygomycetes placed in the order Glomerales. However, the growing of arbuscular mycorrhizae in pure culture in the absence of living host roots is a matter of global concern. Unfortunately, their biotechnological applications cannot be exploited to the level they deserve due to their axenically unculturable nature.

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2 The Medicinal Plants

In the present scenario, herbal medicines are once again gaining popularity as they are easily available, and have rare or no side effects. There is a resurgence in the demands for medicinal herbs. As a consequence, the herbs are now under pressure, also because of shrinking habitats, and are in a phase of extinction. It has, therefore, become necessary to cultivate medicinal plants on a large scale (Rai 1994). At least 20–30 medicinal herbs have been declared chronically endangered by the Government of India. The main reason is increasing biotic pressure on forests and unscientific exploitation of medicinal plants. Conservation of threatened species and promotion of high yielding varieties can be achieved by various modern techniques of biotechnology, such as tissue culture, micropropagation and protoplast culture. Applying these techniques, endangered medicinal plants have been successfully multiplied and developed.

2.1 History of Medicinal Plants

Our ancestors were well equipped with a vast knowledge regarding drugs of natural origin, but they had little knowledge of how to isolate and obtain pure chemical compound as active ingredients. “Charak Samhita” is the oldest text available having a wide resource of hundreds of herbs in the complete treatment of disorders including cholera, tuberculosis, leprosy, etc. “Indian Materia Medica” deals with the detail identification, collection and therapeutic uses of thousands of medicinal plants (Mazumder and Mazumder 2006).

The earliest evidence of humans making use of plants for healing dates back to the Neanderthal period. In the sixteenth century, botanical gardens were created to grow medicinal plants for medical schools. Herbal medicine practice flourished until the seventeenth century when more “scientific” pharmacological remedies were favored.

There are multiple reasons patients turn to herbal therapies. Often cited is a “sense of control”, a mental comfort from taking action, which helps explain why many people taking herbs have diseases that are chronic or incurable, such as diabetes, cancer, arthritis, or AIDS. In such situations, they often believe that conventional medicine has failed them. When patients use home remedies for acute, often self-limited, conditions, such as a cold, sore throat, or bee sting, it is often because professional care is not immediately available, too inconvenient, costly, or time consuming. In rural areas, there are additional cultural factors that encourage the use of botanicals.

Natural plant products are perceived to be healthier than manufactured medicines. Additionally, reports of adverse effects of conventional medications are found in the lay press at a much higher rate than reports of herbal toxicities, in part because mechanisms to track adverse effects exist for conventional medicines whereas such data for self-treatment are harder to ascertain. Even physicians often

dismiss herbs as harmless placebos, and many consumers and physicians alike mistakenly believe that the US Food and Drug Administration (FDA) have not approved anything in a pill form (Winslow and David 1998).

2.2 Herbal Remedies for Human Sufferings

Different surveys conducted in various parts of India revealed that the majority of the people are suffering from different microbial diseases, ranging in severity from mild-like cough or fever to dreadful-like tuberculosis, leprosy, etc. Researchers have found that tendency of self-medication; drug resistance, ignorance, poor health and hygiene are some of the factors responsible for the widespread occurrence of such diseases. Considerable progress has been made during the past two centuries when chemists and biologists accepted the challenge of combating these dreadful diseases by synthesizing a wide plethora of organic compounds having the capacity to combat various pathogens. However, indiscriminate use of synthetic drugs has resulted in mutation of strains making them insensitive to the chemical agent leading to global hazard of drug resistance. The scientists of the twenty-first century are generally reviving our traditional knowledge and are screening various parts of plants scientifically used in folklore medicine in search of newer lead compounds to create alternative medicines (Mazumder and Mazumder 2006).

2.3 Medicinal Importance of Plants

Since ancient times, plants have been an exemplary source of medicine. Ayurveda and other Indian literature mention the use of plants in treatment of various human ailments. India has about 45,000 plant species and, among them, several thousands have been claimed to possess medicinal properties (Grover et al. 2002). The therapeutic value of some of the medicinally important plants is given in Table 1.

2.3.1 Diabetes

Researches conducted in the last few decades on plants mentioned in ancient literature or used traditionally for diabetes have shown anti-diabetic properties as observed by several experimental or clinical data showing anti-diabetic activity. The most effective and the most commonly studied plants in relation to diabetes and their complications are: *Allium cepa, Allium sativum, Aloe vera, Cajanus cajan, Coccinia indica, Caesalpinia bonducella, Ficus bengalenesis, Gymnema sylvestre, Momordica charantia, Ocimum sanctum, Pterocarpus marsupium, Swertia chirayita, Syzigium cumini, Tinospora cordifolia* and *Trigonella foenum*

Table 1 Therapeutic components from plants and their utilization

S. no.	Therapeutic component	Source	Plant part	Market demand for product	Commercial utilization	Remark
1.	Andrographolid	*Andrographic peniculata*	Aerial parts	50%, 95%	Liver diseases treatment	-
2.	Artemisinin	*Artemisia annua*	Aerial parts	99.5%	Anti-malarial	-
3.	Asiaticoside	*Centella asiatica*	Aerial parts	10%, 20%, 99.5%	Skin disorder, Brain tonic, Wound healing,	Herb of Indian origin
5.	Bacosides	*Bacopa monniera*	Aerial parts	20%, 40%	Memory enhancer	-
6.	Berberine	*Berberis aristata*	Roots	99.8%	Anti-inflammatory	-
7.	Boswellic acids	*Boswellia serrata*	Oleo-gum-resin	45%, 65%, 70%	Anti-inflammatory, Anti-arthritic	Free flowing power
8.	Colchicine	*Colchicum luteum*	Seeds	99.5%	Gout, rheumatism	Pharmacopoeal product
9.	Colchicoside	*Colchicum luteum*	Seeds	98.5%	Gout, rheumatism	-
10.	Curcuminoids	*Curcuma longa*	Rhizomes	98.5%	Anti-inflammatory, Cosmetics	in synthesis of THC
11.	Digoxin	*Digitalis lanta*	Leaves	99.5%	Cardiac diseases	Pharmcopoeal product
12.	Forskolin	*Coleus forskohlii*	Root	10%, 20%, 98.5%	Anti obesity, Anti-hypertensive	-
13.	Guggulster-ones (E+Z)	*Commiphora mukul*	Oleo-gum-resin	2.5%	Cholesterol management	Free flowing powder
14.	Gymnemic Acids	*Gymnemia sylvestre*	Leaves	25%, 75%	Anti-diabetic	Gravimetric assay
15.	(-)Hydroxy-citric acid	*Garcinia combogia*	Fruit rind	50%, 70%	Body weight management	-
16.	L-Dopa	*Mucuna pruriens*	Seeds	15%, 30%, 40%	Anti Perkinson & vitality purpose	-
17.	Lutein Esters	*Tagetes eracta*	Flowers	15%, 30%	Anti-oxidant	-
18.	Lycopene	*Lycopersicum esculentum*	Fruit	2%, 5%, 10%	Anti-oxidant	-
19.	Mangostin	*Garcinia mangostana*	Fruit rind	15%, 30%	Anti-oxidant, antibacterial,	-
20.	Reserpine	*Rauwolfia serpentina*	Root bark	98.5%	Anti-hypertensive	-
21.	Sennoside	*Cassia angustifolia*	Leaves, pods	10%, 20%, 40%	Purgative	-
22.	Taxol	*Taxus baccata*	Leaves	99.5%	Breast cancer treatment	-

graecum. All plants have shown varying degrees of hypoglycemic and antihyperglycemic activity (Grover et al. 2002).

2.3.2 Antioxidant Property

Because of increased safety concerns about synthetic antioxidants, exploitation of cheaper and safer sources of antioxidants based on natural origins is the focus of research nowadays (Iqbal et al. 2006).

Plants as antioxidants: there are many plants as antioxidants. Screening of plants is done by measuring the antioxidant activity through various *in vitro* models mice like 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, scavenging of superoxide anion radical-generated non-enzymatic system, ferric thiocyanate method, reducing power, hydrogen peroxide scavenging and metal-chelating activities. For example, *Ocimum basilicum* L. (Lamiaceae) assayed by different methodologies (Gülçin et al. 2007), and black pepper (*Piper nigrum*) (Gülçin 2005).

2.3.3 Organophosphate Exposure

Accidental (environmental or occupational) and self-inflicted (suicide) exposure to organophosphate (OP) pesticides is encountered frequently in the emergency room, especially in the developing world. These perennial public health issues are compounded by a growing concern over the potential use of OP nerve agents such as sarin as a means of terror and nonconventional warfare. OPs disrupt neurotransmission by inhibiting synaptic acetylcholinesterase (AChE-S), leading to an accumulation of acetylcholine in the synapse and neural overstimulation. The severity of the ensuing nicotinic and muscarinic symptoms is dose-dependent and can result in death due to cardiovascular and respiratory collapse. Those surviving often suffer long-term sequelae, including OP-induced delayed neuropathy, muscle weakness, permanent brain dismorphology, and social/behavioral deficits.

For the production of protein pharmaceuticals, plant systems offer low production costs (with comparable purification and regulatory costs), production scalability and flexibility (with low capital investment), and improved safety (no concern of human pathogens and prions).

Nevertheless, mammalian-based production systems seem less promising for large-scale production of AChE-R because of the low levels and relative instability of the protein and its cognate mRNA in such systems. To solve these difficulties, plant-based production is being tried, though still in its experimental infancy, for example: *Nicotiana benthamiana* and endoplasmic reticulum (ER) retention of recombinant human AChE-R. We report here the efficient production and purification of this novel therapeutic protein, a single administration of which provides prophylactic protection from otherwise lethal OP challenges and attenuates the long-term serum AChE-R excess and NMJ damages caused by OP poisoning (Evron et al. 2007).

2.3.4 Anti-carcinogenic Activity

Various components in green and black tea, the beverages made by infusing appropriately processed dried leaves of *Camellia sinensis*, notably simple catechins, have properties *in vitro* that suggest an anti-carcinogenic activity. These include: a direct bactericidal effect against *Streptococcus mutans and S. sobrinus*; prevention of bacterial adherence to teeth; inhibition of glucosyl transferase, thus limiting the biosynthesis of sticky glucan; and inhibition of human and bacterial amylases. Studies in animal models show that these *in-vitro* effects can translate into caries prevention. A limited number of clinical trials in man suggest that regular tea drinking may reduce the incidence and severity of caries. If substantiated, this could offer a very economical public health intervention (Hamilton-Miller 2001). Apart from this, *Withania somnifera* Dunal (Solanaceae) is also under study for anticancer activity (Mathur et al. 2006).

2.3.5 Meticillin-resistant *Staphylococcus aureus* (MRSA)

Meticillin-resistant *Staphylococcus aureus* (MRSA) is recognized as a major nosocomial pathogen that has caused problems in hospitals worldwide, with the UK having one of the highest rates of MRSA in Europe. By far the most important reservoir for MRSA, and hence the most important source for spread and subsequent infection, is patients who may be colonized without evidence of infection. The usual sites of MRSA colonization are areas of broken skin, the groin and the axillae, with MRSA infections occurring most frequently in areas of broken skin and in the bloodstream. It is common practice to attempt to clear MRSA colonization and infection in hospital patients with topical antimicrobials and antiseptics; mupirocin and chlorhexidine, for example, are currently employed as part of standard hospital MRSA decolonization protocols. However, resistance to these agents is increasing, with a marked increase in antibiotic resistance recently reported for bacterial strains isolated from superficial skin wounds and leg ulcers. Alternative agents for MRSA decolonization are therefore required.

Tea-tree oil (TTO), the essential oil of *Melaleuca alternifolia*, has been suggested as a potential agent for MRSA decolonization, as it has been shown to be an effective broad-spectrum anti-microbial with good activity *in vitro* against a variety of bacteria including MRSA. Furthermore, it has been shown that bacteria such as *S. aureus* that transiently colonize the skin were more susceptible to TTO than bacteria such as coagulase-negative staphylococci (CoNS), which are regarded as part of the normal commensal skin flora. It has been suggested, therefore, that TTO could be useful for removing transient skin flora while suppressing but still maintaining the resident flora, which acts as a natural defence against colonization by other pathogenic bacteria. Studies comparing the activity of TTO against planktonically grown clinical skin isolates of MRSA, meticillin-sensitive *S. aureus* (MSSA) and CoNS using both a modified broth microdilution method and a quantitative *in vitro* time–kill test method have been carried out (Pinto et al. 2006).

2.3.6 Candiasis

Fungal infections have been increasing in recent years due to a growing number of high-risk patients, particularly immunocompromised hosts. *Candida* is the third- or fourth-most common isolate in nosocomial bloodstream infections in the USA. In addition, candidosis is the most common invasive fungal infection in critically ill nonneutropenic patients.The mortality rate due to invasive aspergillosis increased by 357% between 1980 and 1997 in the USA. Dermatomycoses are common infections caused by members of the genus *Candida* and by filamentous fungi, particularly the dermatophytes. Superficial candidosis and dermatophytosis can be severe in immunocompromised patients.

In spite of the introduction of new antifungal drugs, they are limited in number. The increase of fungal resistance to classical drugs, the treatment costs, and the fact that most available anti-fungal drugs have only fungistatic activity, justify the search for new strategies.

Aromatic plants have been widely used in folk medicine. It is known that most of their properties are due to their volatile oils. Essential oils from many plants are known to possess antifungal activity, but only limited information exists about activity towards human fungal pathogens. They have been empirically used as antimicrobial agents, but the mechanisms of action are still unknown.

According to our preliminary results some essential oils show an important antifungal activity against yeasts, dermatophyte fungi and *Aspergillus* strains, which could predict therapeutic benefits, mainly for diseases with mucosal, cutaneous and respiratory tract involvement.

Several studies have shown that thyme oils, particularly those of *Thymus vulgaris* and *T. zygis* possess antimicrobial activity, those of the phenol type being the most active. The limited occurrence of these phenols in nature is one of the reasons why *Thymus* oils containing thymol and carvacrol have been of great interest for some time.

Thymus pulegioides is widely distributed on the European continent south of the Mediterranean islands. In Portugal, it grows in the northeast, and it is locally used as an antiseptic. Previous results have demonstrated that this species is polymorphic, and that the thymol/carvacrol chemotype is one of the most abundant in Portugal (Pinto et al. 2006).

2.3.7 Plants Showing Anti-fungal Properties

The essential oils of *Origanum vulgare* ssp. *hirtum, Mentha spicata, Lavandula angustifolia*, and *Salvia fruticosa* exhibited antifungal properties against the human pathogens *Malassezia furfur, Trichophyton rubrum*, and *Trichosporon beigelii*. Of the four oils, *O. vulgare* ssp. *hirtum* oil showed the highest fungicidal activity and at a dilution of 1/50,000 caused a 95% reduction in the number of metabolically active cells within 6 h of exposure. Among the main components of the four oils, carvacrol and thymol exhibited the highest levels of antifungal activity. The therapeutic

efficacy of the *O. vulgare* ssp. *hirtum* essential oil was tested in rats experimentally infected with *T. rubrum* and yielded promising results. Furthermore, the above essential oils were tested with the Ames test and did not exhibit any mutagenic activity (Adam et al. 1998).

2.3.8 Plants Showing Contraceptive Effects

Since early times, plants were used to control fertility, but now this knowledge is restored only to the tribal population. The prosecution of witches in early modern Europe led to the decline of "wise women", who had for centuries transmitted the lore of contraception. By the seventeenth and eighteenth centuries, that knowledge was everywhere disappearing from Europe, and it remained for researchers in the twentieth century to rediscover it. There is an absence of evidence of the widespread use of effective contraceptives before the modern era. It is assumed that, because of social constraints, knowledge of contraception remained a secret lore, which was transmitted orally or alluded to in written sources in coded form (Riddle 1997).

But this knowledge is under study again and the following are a few of the plants which are being studied for their contraceptive property: *Ancistrophyllum secundiflorum* (Odesanmi et al. 2002), *Tripterygium wilfordii*, a Chinese herbal plant (Kutney et al.1992), Neem oil from *Azadirachta indica* (Juneja et al.1994), *Emblia ribes* (Williamson 2002), *Montanoa tomentosa* (Browner and Bernard 1986), *Carica papaya* (Lohiya et al. 1994), *Trigonella foenum graecum* (Fenugreek) (Kassem et al. 2006), *Vicoa indica* (Banjauri) (Dhall and Dogra 1988), and *Gloriosa superba* (Dixit et al. 1983).

2.4 Regulation: Dietary Supplement and Health Education Act

In 1993, the FDA began scrutinizing the herbal and supplement industry, which triggered a massive letter-writing campaign organized by health food stores. Under pressure, the FDA created the supplement category, which includes vitamins, minerals, and herbs, and created the Dietary Supplement and Health Education Act (DSHEA), signed October 1994. The DSHEA requires no proof of efficacy, no proof of safety, and sets no standards for quality control for products labeled as supplements. Although the DSHEA requires that supplements do not promise a specific cure on the label, they may claim an effect. Now, if questions arise, the burden lies with the FDA to prove a product unsafe, rather than a company proving its product safe. Manufacturers must put a message on the label stating that the FDA has not reviewed claims, but this statement can be subtle. In contrast, regulating agencies in Germany, France, the United Kingdom, and Canada enforce standards of herb quality and safety assessment on manufacturers.

Because of the lack of requirements for quality control, safety, and efficacy, consumers cannot determine if a herb's active ingredients are actually in the product, if the ingredient is bioavailable, if the dosage is appropriate, if the next bottle

they buy will have the same components, or what else is in the pill besides the claimed ingredients (Winslow and David 1998).

2.5 Present Status of Herbal Medicines

The age-old system of medicine has been neglected mainly because of the rapid expansion of allopathic medical treatment. Presently, the Indian system of medicine uses over 1,100 medicinal plants and most of them are collected regularly from the wild, of which over five dozen species are said to be in great demand (Mazumder and Mazumder 2006).

2.6 Future Prospects and Constraints of Herbal Drug Industry

An upsurge in the use of products based on plants is booming. Medicinal plants and their derivatives will continue to play a major role in medical therapy in spite of advances in chemical technology and the appearance of cheap, synthesized, complex molecules from simple ones through highly specific reaction mechanisms. The reactions involved are either difficult or expensive to duplicate by classical chemical methods.

Since production of drugs from medical plants is less expensive than chemical synthesis (Mazumder and Mazumder 2006) and other associated benefits, the use of herbal products is going to increase not just nationally but internationally, requiring a need for the conservation of natural flora. Along with the flourishing herbal industry there is an urgent need to develop ethics for the use of herbs. The world is already facing issues like global warming and deterioration of the natural environment, so if the herbal industry is to be promoted to the desired level there will be a requirement to set up stringent rules and regulations for the conservation of flora and fauna. We are facing problems in the conservation of endangered species and, with the advent of massive herbal production, the current scenario is getting worse. There is a need to develop techniques to enhance the active ingredient from its usual quantity present in the plant, and to enhance the biomass and better growth of medicinally important herbs.

3 *Piriformospora indica* – Model Symbiotic Fungus

Varma and his collaborators, from the School of Life Sciences, Jawaharlal Nehru University, New Delhi, have screened a novel endophytic root-colonizing fungus which mimics the capabilities of a typical AM fungus. However, the unique feature is that this fungus is axenically culturable, and this is a golden lining for AM

fungi for the scientist dealing with the mycorrhizal research. The fungus has been named *Piriformospora indica* based on its characteristic pear-shaped chlamydospores (Fig. 1), and is related to the Hymenomycetes of the Basidiomycota (Verma et al. 1998).

Piriformospora indica tremendously improves the growth and overall biomass production of diverse hosts, including legumes (Varma et al. 1999, 2001), medicinal and economically important plants (Rai et al. 2001; Peškan-Berghöfer et al. 2004; Rai and Varma 2005; Shahollari et al. 2005; Prasad et al. 2007). A pronounced growth-promoting effect was seen with terrestrial orchids (Blechert et al. 1999; Bhatnagar and Varma 2006). A study suggested that *P. indica* is able to colonize the rhizoids of liverworts and that the thalli failed to grow under in situ conditions in the absence of this fungus (Varma et al. 2000). The fungus also provides protection when inoculated into the tissue culture-raised plants by overcoming the 'transient transplant shock' on transfer to the field, and provides almost 100% survival on transplant (Sahay and Varma 1999, 2000). Based on anatomical and genomic studies, *P. indica* has been attributed to the highly evolved Hymenomycetes (Basidiomycetes) (Fig. 2).

However, neither clamp connections nor sexual structures could be observed. The morphological features and 18S gene sequences certainly placed the fungus in the group. The septal pores consisted of dolipores with continuous parenthosomes. The dolipores were very prominent, with a multilayered cross wall. The parenthosomes were in contact with the ER membranes, which were mostly found near the dolipore (Verma et al. 1998).

The fungus colonizes the roots and improves the health, vigor and survival of a wide range of mono-and dicotyledonous plants. This fungus grows on a large varieties of inorganic, organic and polyphosphates, and thus serves as a good model organism to study phosphorus metabolism (Malla et al. 2004). The molecular mass

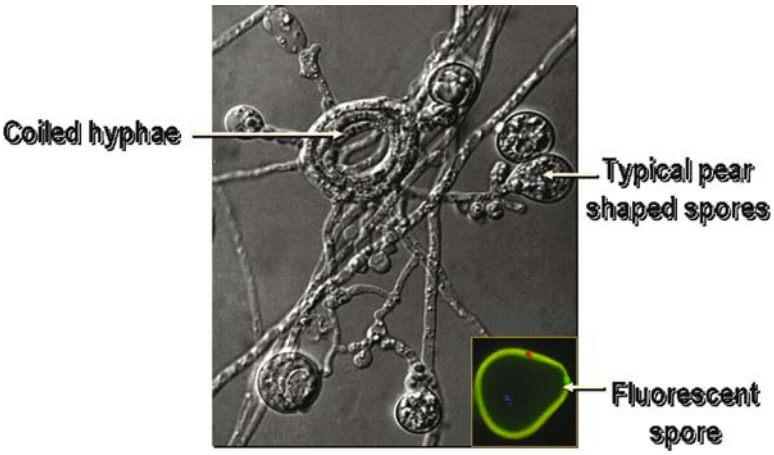

Fig. 1 Electron micrograph of *Piriformospora indica* showing typical coiling and pear shaped spores

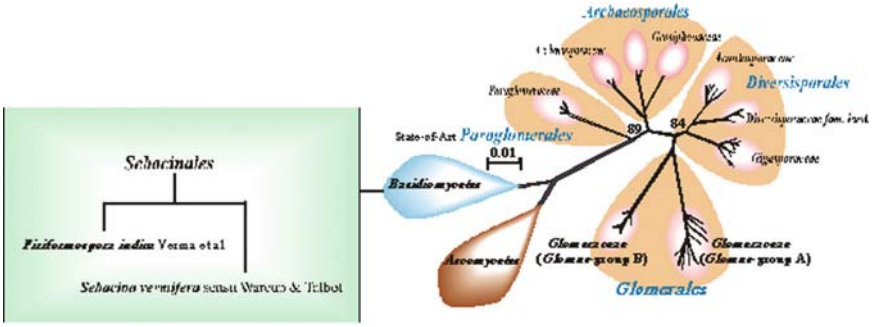

Fig. 2 Proposed generalised taxonomic structure of the AM and related fungi (Glomeromycota), based on SSU rRNA gene sequences. *Thick lines* delineate bootstrap support above 95%, lower values are given on the branches. The four-order structure for the phylogenetic position of *P. indica* (after Schüßler et al. 2001)

of denatured acid phosphatase (ACPase) of *P. indica* was found to be 66 kDa on SDS PAGE. This fungus mediates uptake of phosphorus from the substratum and its translocation to the host by an energy-dependent active process, serves as a strong agent for biological hardening of tissue culture-raised plants, protecting them from "transplantation shock", and renders almost 100% survival rate on the hosts tested. This fungus is also a potential "bio-control agent" against potent root pathogens. Thus, it displays immense potential to be utilized as a biological tool for plant promotion, protection from pests, and for relieving stress conditions such as those due to acidity, desiccation and heavy metal toxicity. Thus, it may be concluded that this novel fungus has immense potential for biotechnological applications.

3.1 Phylogenetic Position of P. indica

Recently, molecular techniques like polymerase chain reaction (PCR), molecular cloning, and sequencing showed that members of *Sebacinaceae* have been involved in various mycorrhizal associations. Proteomics and genomics data about this fungus has recently been described (Peškan-Berghöfer et al. 2004; Kaldorf et al. 2005; Shahollari et al. 2005). However, sebacinoids were demonstrated recently to be ectomycorrhizal (Selosse et al. 2002). Observations on ectomycorrhizae and basidiomes suggest that species of *Sebacinaceae* are fairly common mycobionts in various ectomycorrhizal plant communities (Urban et al. 2003). The phylogenetic position of the *Sebacinaceae* within the Basidiomycota gives an overview of phylogenetic relationships inside this subgroup of Hymenomycetes for which the new order *Sebacinales* is proposed. The ultrastructural data also indicate that *P. indica* is a member of the Hymenomycetes (Basidiomycota), and studies on the molecular phylogeny will help to reveal the closest relatives of this species (Fig. 3).

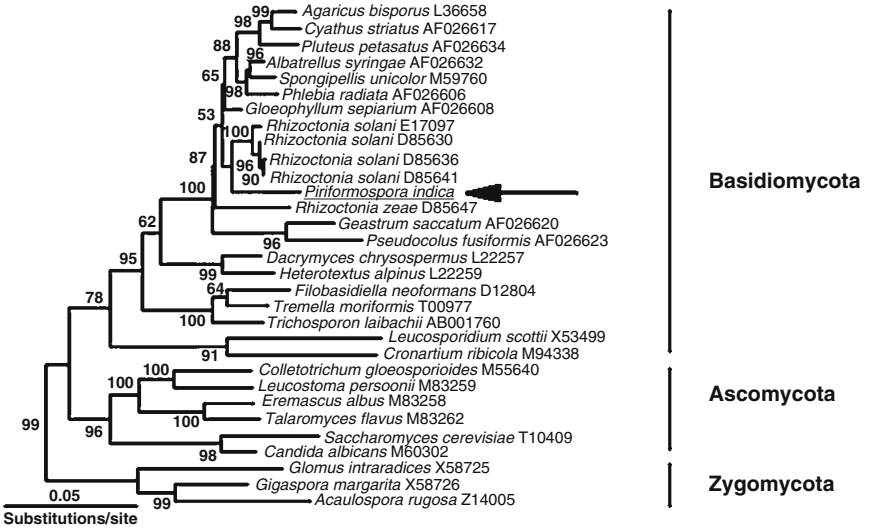

Fig. 3 Maximum-likelihood tree estimated by quartet puzzling method (Strimmer and Haeseler, 1996) on 18s rDNA sequences showing phylogenetic relationships between *P. indica* and other representatives of Basidiomycetes by Verma et al. (1998)

Immunological characterization showed its strong cross-reactivity with the members of Zygomycota (*Glomerales*) instead of Basidiomycota (Varma et al. 2001; Singh et al. 2003b), which needs further critical appraisal.

A neighbor-joining analysis on comparisons of partial 18s rDNA sequences (525 nucleotide position) placed *P. indica* close to the *Rhizoctonia solani* group (*Ceratobasidiales*) within the Basidiomycota. A maximum-likelihood analysis on complete 18s rDNA sequences (1,550 nucleotide positions) confirmed this finding. A comprehensive phylogenetic analysis of *Rhizoctonia* using sequences from mitochondrial and nuclear rDNA on more representatives may provide an insight into the evolution of this important group and its evolutionary relationship with *P. indica* within Hymenomycetes. Analysis of 28s rDNA exhibited no change with respect to the taxonomic status of *P. indica* (Varma et al. 2001). Thus, based on the 18s and 28s rDNA analysis and the ultrastructure of the septal pore, it is placed within the Hymenomycetes (Basidiomycota).

3.2 Applications and Diverse Functions

The fungus *P. indica* associates with the roots of various plant species in a manner similar to mycorrhiza and promotes their growth (Varma et al. 1999, 2001; Singh et al. 2002, 2003a; Pešken-Berghöfer et al. 2004; Pham et al. 2004a; Oelmüller et al. 2004, 2005; Shahollari et al. 2005; Deshmukh et al. 2006). The fungus possesses unique properties to act as biofertilizer, bioprotector and immunoregulator.

It also plays a key role in protecting roots from insects by increasing the tolerance of the host roots (Varma et al. 1999; Waller et al. 2005; Serfling et al. 2007). It also promotes the antifungal potential of the medicinal plant *Spilanthus calva* due to an increase in spilanthol content after interaction (Rai et al. 2004).

Among the compounds released in root exudates infected with *P. indica*, flavonoids are found to be present. Flavonoids have been suggested to be involved in stimulation of precontact hyphal growth and branching (Gianinazzi-Pearson et al. 1989; Siqueira et al. 1991), which is consistent with their role as signaling molecules in other plant–microbe interactions (Giovannetti and Sbrana, 1998). Cell wall degrading enzymes like cellulase, polygalactouronase and xylanase were found in significant quantities both in the culture filtrate and in the root exudates colonized by *P. indica*.

P. indica showed profound effects on disease control when challenged with a virulent root and seed pathogen, *Gaeumannomyces graminis*, by completely inhibiting the growth of this pathogen. It indicates that *P. indica* acted as a potential agent for biological control of root diseases, although the chemical nature of the inhibitory factor is still unknown (Varma et al. 2001).

3.3 Eco-Functional Identity

P. indica colonizes the root cortex and forms inter- and intracellular hyphae. Within the cortical cells, the fungus often forms dense hyphal coils or branched structures intracellularly. The fungus also forms spore- or vesicle-like structures within or between the cortical cells. Like AM, hyphae multiply within the host cortical tissues and never traverse through the endodermis. Likewise, they also do not invade the aerial portion of the plant (stem and leaves). However, under certain modified cultural conditions, fungus may also invade the stem and leaves without damaging the plant. The characteristic features of *P. indica* are axenically culturable: no clamp connections, anastomosis present, hypha–hypha aggregation, no hyphal knots, simple septum with dolipores and continuous, straight parenthosomes, chlamydospores 16–25 µm in length and 10–17 µm in width and 8–25 nuclei per spore

3.4 Host Spectrum

The host spectrum of *P. indica* is very much like AM fungi: it has been calculated that AM fungi interact with almost 90% of the terrestrial plants (Bagyaraj and Varma, 1995; Giovannetti and Sbrana 1998; Smith and Read, 1997; Varma et al. 1999). However, only limited members of the plant community have failed to interact and these belong to the family of Amaranthaceae, Chenopodiaceae, Cyperaceae, Junaceae, Proteaceae, or lupines and Cruciferae, etc. (Denison et al. 2003). A careful

perusal of the literature indicates that this statement may not be true (Leake 1994; Tester et al. 1987). Denison et al. (2003) have emphasized that model systems are also important as a new research tool to understand the co-operation between microbes and the plants. Cruciferae includes the model plant, *Arabidopsis thaliana*, that lacks symbiotic interactions such as mycorrhizae and rhizobia. However, most species of plants are normally infected by mycorrhizae, but some plant taxa do not usually form recognizable mycorrhization.

P. indica colonizes the roots of host plants of diverse groups of economically important crops: medicinal (Rai et al. 2001, 2004), horticultural, forest and ornamental plants (Varma et al. 1999, 2001). The similar host range of *P. indica* and AM fungi suggests that this phenomenon may be correlated with some identical functional aspects as indicated by the serological data (ELISA, Western blotting, immunofluorescens and immunogold labeling) showing close similarities between AMF and *P. indica* (Singh et al. 2003b; Varma et al. 1999).

One of the striking differences is that, unlike AM, the host range of *P. indica* also includes terrestrial orchids *Dactylorhiza purpurella* (T. & T.A. Stephenson), Soo, *D. incarnata* (L.) Soo, *D. majalis* (Rchb.) P.F. Hunt & Summerh. and *D. fuchsii* (Druce) Soo (Blechert et al. 1999; Singh and Varma 2000; Singh et al. 2001; Varma et al. 2001) (Table 2).

However, exceptions are those belonging to members of the Cruciferae and some members of Chenopodiaceae and Amaranthaceae (Read 1999; Varma et al. 2001). Literature reports that the members of these group normally do not accept

Table 2 Representative species of some families which interact with *Sebacinales*

Family	Genera
Bryophyte (liverwort)	*Aneura pinguis* L. Dumort. (liverwort)
Acanthaceae	*Adhatoda vasica* L. syn. (malabar nut)
Apiaceae (Umbelliferae)	*Daucus carota* L. Queen Anne's-lace (carrot)
Asteraceae	*Artemisia annua* L. (chinese wormwood)
Asteraceae	*Spilanthes calva* DC (clove)
Brassicaceae	*Arabidopsis thaliana* L. Heynh. (mouse ear cress)
Combretaceae	*Terminalia arjuna* L. (Arjun tree/stembark)
Fabaceae (Mimosoideae)	*Acacia catechu* (L.f.) Willd (black catechu)
Fabaceae	*Glycine max* L. Merr. (soybean)
Fagaceae (oak family)	*Quercus robur* L. (clone DF 159) (oak)
Liliaceae	*Chlorophytum borivillianum* Baker (musli)
Meliaceae	*Azadirachta indica* A. Juss (neem)
Orchidaceae	*Dactylorhiza fuchsi* Druce (Soo') (spotted orchid)
Poaceae	*Oryza sativa* L. (rice)
Rhamanaceae	*Zizyphus nummularia* Burm. fil. (jujube)
Rubiaceae	*Coffea arabica* L. (English coffee)
Salicaceae	*Populus tremula* L. (aspen)
Scrophulariaceae	*Bacopa monniera* L. Wett. (brahmi)
Solanaceae	*Nicotiana tobaccum* L. (tobacco)
Solanaceae	*Withania somnifera* L. Dunal (winter cherry)
Verbenaceae	*Tectona grandis* Linn. f. (teak)

AM fungi. *In vitro* studies, on *P. indica* and *S. vermifera* sensu recorded that these two symbiotic fungi profusely interacted with the root system of the crucifer plants viz., mustard (*Brassica junacea*), spinach (*Spinaceae oleracea*), cabbage (*Brassica oleracea* var *capitata*) (Kumari et al. 2003) and *Arabidopsis thaliana* (Pham et al. 2004a; Peškan-Berghöfer et al. 2004; Shahollari et al. 2005). It would be useful to assess the non hosts of AM fungi with respect to their interaction with *P. indica* for its further functional characterization. In order to enlighten the molecular events that promote the root growth, the difference in protein expression was analyzed and modification arises due to the interaction with the fungus. Membrane-associated proteins from roots were separated by two-dimensional gel-electrophoresis (2D-PAGE) and identified by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (MS-MS). *P. indica* consists of secondary metabolites like hydroxamic acids (DIBOA, DIMBOA) which act as natural pesticides (Varma et al. 2001).

4 Interaction Between Novel Symbiotic Fungus *P. indica* and Medicinal Plants

P. indica tremendously improves the growth and overall biomass production of diverse hosts, including legumes (Varma et al. 1999, 2001), medicinal and other economically important plants (Pham et al. 2004b; Rai et al. 2001; Peškan-Berghöfer et al. 2004; Shahollari et al. 2005). *P. indica* colonizes the roots of host plants of a diverse group of plants belonging to monocots, dicots including orchids (Blechert et al. 1999: Pham et al. 2004b, Prasad et al. 2005), herbs, shrubs and woody trees. The effect of *P. indica* interaction with various plants such as *Bacopa monniera, Azadirachta indica, Tridex procumbans, Abrus precatorius, Withania somnifera, Chlorophytum borivilianum* and *Spilanthes calva* (Rai et al. 2001, 2004) have been tested in laboratory conditions as well as in the extensive field trial.

4.1 Spilanthes calva

Spilanthes calva DC (Family Asteraceae), commonly known as toothache plant or virus blocker, is well known for enhancing immunity. Because of its high medicinal value, it is costly and there is much demand of this plant in the market. It is cultivated in tribal pockets for herbal treatment in various diseases. This plant has antiageing properties and cures various diseases of tooth and gums including pyorrhoea. It is antimicrobial in nature and economically very useful as tooth powder, which is prepared from this plant (Dey 1980) Its leaves stimulate salivation, which is due to the presence of an active chemical spilanthol. Manifold enhancement of the antifungal activity and quantity of spilanthol was recorded on cocultivation with *P. indica* (Fig. 4). The chemical analysis of the roots of the plant revealed a slight increase in spilanthol content.

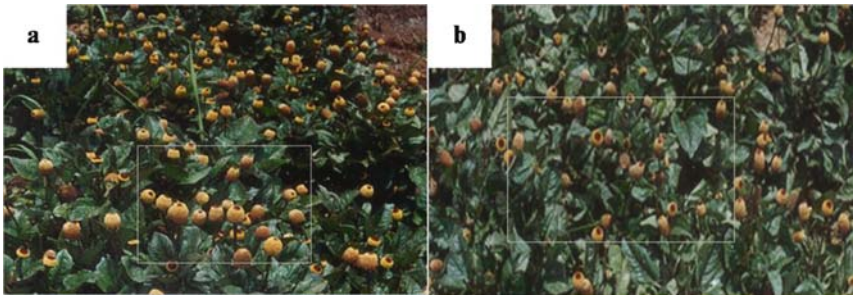

Fig. 4 A field trial of *Spilanthus calva* in Central India. **a** Cocultivation with *P. indica*; **b** control

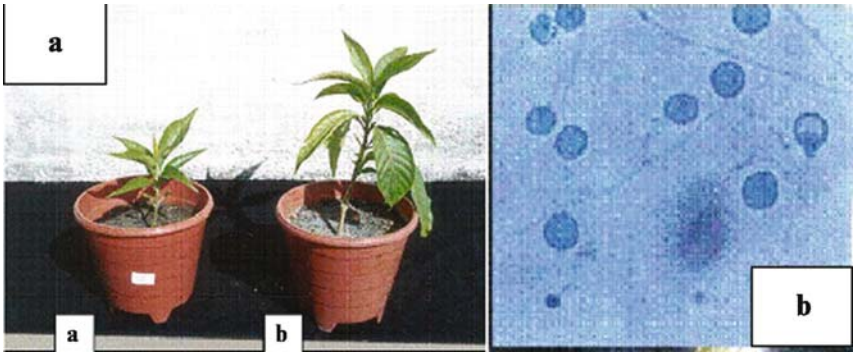

Fig. 5 Growth promotional effect of *P. indica* on medicinal plant *Adhatoda vasica*. **a** *a* Control, *b* inoculated with *P. indica*; **b** micrograph of colonized root of *Adhatoda vasica*

4.2 Adhatoda vasica

Adhatoda vasica Nees (common name, Malabar nut; family, Acanthaceae) is an evergreen shrub. It is well known for preparation of medicine for bronchitis, asthma and other pulmonary infections. *Glycodin*®, a famous product used for the cure of bronchitis, is extracted from the leaves of this plant. It is also known for its antiarthritis, antiseptic, antimicrobial, expectorant, sedative and antituberculosis properties (Dey 1980; Singh and Jain 1987). In Ayurveda, several medicines are manufactured from this plant. Due to increasing demand for *A. vasica* by pharmacies, there is a need for its rapid multiplication. In the observations, cuttings of *A. vasica* were inoculated with *P. indica* to assess the growth-promoting property of *P. indica* on this important medicinal plant. Profuse proliferation of roots of *A. vasica* after inoculation of *P. indica* was repeatedly recorded (Fig. 5). Root-colonization of *A. vasica* by *P. indica* increased with time from 53% after 2 months to 95% after 6 months (Rai and Varma 2005).

4.3 Withania somnifera

Withania somnifera is also known as Indian Ginseng and belongs to the family Solanaceae. More than 91 pharmaceutical products are produced from the roots of this plant. Multiple shoot cultures of *Withania somnifera* were established from single shoot tip explants and their potential for the production of two principal withanolides, withaferin A and withanolide D, was investigated (Ray and Jay 2001; Ganzera et al. 2003). The plantlets were then transferred to pots and maintained in a greenhouse for 4 months. 90% of these *in vitro*-propagated plantlets survived and showed normal growth. Leaves from these plants were used for isolation of the withanolides. Methanolic extract of leaves from plantlets growing in tissue culture and those transferred to the greenhouse were evaluated for immunomodulatory activity. While the extract from greenhouse samples showed potent immunosuppressive activity, those from tissue culture samples did not show any activity (Furmanowa et al. 2001). Withaferin A acts as radiosensitizer from *Withania somnifera*. SER (sensitizer enhancement ratio) increased with drug dose, but at higher doses the increased lethality appears to be due to two effects, drug toxicity and radiosensitization The applicability of this drug as a radiosensitizer in cancer therapy needs to be explored (Devi et al.1996; Devi 1996).

The basal stem and leaf areas of treated plants were also enhanced. The lengths of the inflorescence and the number of flowers on inoculated *S. calva* plants were also increased relative to controls. Similarly, the number of flowers on the flowers on the inoculated plants of *W. somnifera* was higher than on controls. Seed counts were higher for treated than for control plants.

The overall root biomass of the inoculated plants was higher than that of the corresponding controls. The fresh and dry weights of both underground and aboveground parts of *W. somnifera* inoculated plants were higher than controls (Fig. 6). The net primary productivity of inoculated *S. calva* and *W. somnifera* plants was 0.06 and 0.23 g/plant/day, respectively. These values were higher than those of control plants (0.02 and 0.12 g/plant/day, respectively).

4.4 Safed Musli

Safed musli, scientifically known as *Chlorophytum borivilianum*, belongs to the Liliaceae family and is endowed with Rasayana (antiageing and immunoboosting), Balya (performance-boosting) and Vrishya (aphrodisiac) properties to keep one young and healthy with a well-tuned body for better handling of stress. It lives up to the description as recent clinical trials show that musli if taken on regular basis, it maintains health and youthfulness, keeps the person energetic and active, increases working capacity, increases tolerance to stress and strain, increases working capacity, gives sound sleep, develops a firm and muscular body, and improves conjugal capability. Phytochemicals like saponins, carbohydrate and proteins are

Fig. 6 Biological hardening of *Withania somniferum* with *P. indica*

present in the root. This plant has got immense market potential, both domestic and international. Peeled and dried roots are used for therapeutic purposes. Both tubers and seeds are used, but the tubers are a more viable option because they offer better germination rate and tuber growth than seeds. They are normally used to maintain the equilibrium of all the systems of the body and keeps the "Body-Mind-Soul Complex" in a state of harmony. On interaction with *P. indica*, significant growth promotional effect on the plant has been observed besides early flowering in the crop and 90% survival on transplantation (Fig. 7).

4.5 Bacopa monniera

Bacopa monniera commonly known in India as Brahmi is an important ancient Ayurvedic medicinal plant in the Scrophulariaceae family. In the traditional system of medicine, Brahmi is a reputed nervine tonic. It is also used to treat asthma, insanity, epilepsy, hoarseness, enlargement of the spleen, snake bite, rheumatism, leprosy, eczema and ringworm, and as a diuretic, aperitive and cardiotonic (Basu and Walia 1944; Basu et al. 1967; Bhakuni et al. 1969; Elangovan et al. 1995). The main active ingredient of *B. monniera* is believed to be the bacosides.

The fungus *P. indica* is documented to promote plant growth and protects the host against root pathogens and insects. Fungus root colonization by the fungus *P. indica* promoted the plant growth and enhanced antioxidant activity as well as the active ingredient bacoside by several folds (Fig. 8).

Fig. 7 >90% survival rate, establishment and full development of micropropagated plants after treatment with *P. indica*

Fig. 8 Significant growth promotional effect of *P. indica* on *Bacopa monniera* plant in tissue culture medium. **a** Control; **b** cocultivated with *P. indica*

Tissue culture technology could play an important role in the clonal propagation, germplasm conservation and improvement of *B. monniera*. Shoot regeneration has been reported from the distal ends of 1- to 12-mm-long internode segments of *B. monniera* cultured on growth regulator free medium, longer internodes being more conducive to regeneration (Thakur et al. 1976; Tiwari et al. 1998).

It was found that there was an inherent problem with the micropropagated plants during the time of transplantation from the laboratory to field. It was noticed that the rate of survival was very low, up to 40% in the field conditions. The salient reason could be the 'transient transplant shock' that resulted in the stunted growth. Biological hardening has proved to be fruitful. It provided better results for the overall performance of the plants. For employing the technique, inoculation of micropropagated plantlets with active cultures of AMF or mycorrhiza-like fungi appears to be critical for their survival and growth. These preacclimatized plantlets, when transferred to the field, overcame the transient transplant shock, and were able to cope with the changed environment of transplantation which also helped in their successful establishment. Pre-establishment of mycorrhiza in the host roots also helps in the development of the synergistic effect with other rhizosphere microflora that compete in the ecosystem for successful survival (Prasad et al. 2004).

Conclusion

Medicinal plants are in great demand in modern civilization to extract various herbal drugs for human welfare. These products come from a labor- and capital-intensive activity, where chemical inputs play an essential role but bring with them a set of problems linked to the degradation of the natural environment and resource base. Thus, the potential use of biological tools such as micropropagation and biological hardening with AM, which ensure adequate level of production with satisfactorily reduction of chemical fertilizer and pesticides, like technologies needed for sustainable agriculture. Among the soil inhabiting microorganisms, AM and other mycorrhizae-like fungi acquire added importance due to their role in establishment, productivity and longevity of natural and man-altered ecosystems. AM fungi share A distinct ecological niche in soil along with a variety of microorganisms including some which are pathogenic, some commensalistic and some which are symbiotic.

A newly-described species *Piriformospora indica* covers most of the characteristics of AM. It improves the growth and overall biomass production of a diverse host including medicinal and other plants of economic importance.

This fungus has another important feature that it has potency to grow axenically as an effective alternative to AM. This fungus mediates uptake of phosphorus from the substratum and it is translocated to the host by an energy-dependent active process. The fungus serves as a strong agent for biological hardening of tissue culture-raised plants, protecting them from "transplantation shock", and rendering almost 100% survivals on the hosts tested. This fungus is also a potential "biological agent" against potent root pathogens. The growth promotion observed may have

been caused by a greater absorption of water and mineral nutrients due to extensive root colonization and the proliferation of the mycelium into the soil. Thus, it can be concluded that this root-colonizing fungus promotes growth of many plant species of medicinal and economical importance, including cereals, legumes, ornamental plants, oilseed and vegetables, is a potential candidate for the hardening of tissue culture-raised plants and exerts fungicidal and herbicidal resistance. In plant biotechnology, the emphasis is on the manpower rather than on expensive equipment in both developing and developed countries because of its vast impact on agriculture. Today, micropropagation is the most widely and successfully used technology for the mass production of horticultural, ornamentals, fruits, vegetable, cereals, plantation crops, spices and medicinal plants.

References

Adam K, Sivropoulou A, Kokkini S, Lanaras T, Arsenakis M (1998) Antifungal activities of *Origanum vulgare* sub sp. *hirtum, Mentha spicata, Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. J Agric Food Chem 46: 1739 –1745

Bagyaraj DJ, Varma A (1995) Interaction between arbuscular mycorrhizal fungi and plants, and their importance in sustainable agriculture in arid and semi–arid tropics. In: Jones JG (ed) Advances in microbial ecology, vol 14. Plenum, NewYork, pp 119–142

Basu N, Rastogi RP, Dhar ML (1967) Chemical examination of *Bacopa monniera* Wettst. Part III: bacoside B. Indian J Chem 5: 84–86

Basu NK, Walia JS (1944) The Chemical investigation of the leaves of *Herpestis monniera*. Indian J Pharm 4: 84–91

Bhakuni Ds, Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN (1969) Screening of Indian plants for biological activity. Part II. Indian J Exp Biol 7: 398–402

Bhatnagar K, Varma A (2006) The healthy marriage between terrestrial orchids and fungi. J Hill Res India 19: 1–12

Blechert O, Kost G, Hassel A, Rexer RH, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchids. In: Varma A, Hock B (eds) Mycorrhiza, 2nd edn. Springer, Heidelberg, pp 683–688

Browner CH, Bernard ROM (1986) Herbal emmenagogues used by women in Colombia and Mexico. In: Etkin NL (ed) Plants in indigenous medicine & diet: bio-behavioral approaches. Routledge, London, pp 32–34

Denison RD, Bledsoe C, Kahn M, Gara FO, Simms EL, Thomashow LS (2003) Cooperation in the rhizosphere and the "free rider" problem. Ecology 84: 838–845

Deshmukh S, Huckelhoven R, Schafer P, Imani J, Sharma M, Weiss M, Waller F, Kogel KH (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103: 18450–18457

Devi PU (1996) *Withania somnifera* Dunal (Ashwagandha): potential plant source of a promising drug for cancer chemotherapy and radiosensitization. Indian J Exp Biol 34: 927–32

Devi PU, Akagi K, Ostapenko V, Tanaka Y, Sugahara T (1996) Withaferin A, a new radiosensitizer from the Indian medicinal plant *Withania somnifera*. Int J Radiat Biol 69: 193–197

Dey AC (1980) Indian medicinal plants used in Ayurvedic preparations. Bishen Singh Mahendra Pal Singh, Dehradun

Dhall K, Dogra M (1988) Clinical trials with *Vicoa indica* (Banjauri), an herbal medicine, as an antifertility agent. Contraception 37: 75–84

Dixit VP, Joshi S, Kumar K (1983) Possible antispermatogenic effect of *G. superba* in male gerberils. Comp Physiol 8:17–22

Elangovan V, Govindasamy S, Ramamoorthy N, Balasubramanian K (1995) *In vitro* studies on the anticancer activity of *Bacopa monnieri*. Fitoterapia 66: 211–215

Evron,T, Geyer BC, Cherni I, Muralidharan M, Kilbourne J, Fletcher SP, Soreq H, Mor TS (2007) Plant-derived human acetylcholinesterase-R provides protection from lethal organophosphate poisoning and its chronic aftermath. FASEB J 21: 2961–2969

Furmanowa M, Gajdzis-Kuls D, Ruszkowska J, Czarnocki Z, Obidoska G, Sadowska A, Rani R, Upadhyay SN (2001) *In vitro* propagation of *Withania somnifera* and isolation of withanolides with immunosuppressive activity. Planta 67: 146–149

Ganzera M, Choudhary MI, Khan IA (2003) Quantitative HPLC analysis of withanolides in *Withania somnifera*. Fitoterapia 74: 68–76

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7: 243–255

Giovannetti M, Sbrana C (1998) Meeting a non-host: the behaviour of AM fungi. Mycorrhiza 8: 123–130

Grover JK, Yadav S, Vats VJ (2002) Medicinal plants of India with anti-diabetic potential. J Ethnopharmacol 81: 81–100

Gülçin I (2005) The antioxidant and radical scavenging activities of black pepper (*Piper nigrum*) seeds. Int J Food Sci Nutr 56: 491–9

Gülçin I, Elmastas M, Aboul-Enein HY (2007) Determination of antioxidant and radical scavenging activity of Basil (*Ocimum basilicum* L. Family Lamiaceae) assayed by different methodologies. Phytother Res 21: 354–61

Hamilton-Miller JMT (2001) Anti-cariogenic properties of tea (*Camellia sinensis*). J Med Microbiol 50: 299–302

Iqbal S, Bhanger MI, Akhtar M, Anwar F, Ahmed KR Anwer T (2006) Antioxidant properties of methanolic extracts from leaves of *Rhazya stricta*. J Med Food. 9: 270–5

Juneja SC, Pfeifer T, Williams SR, Chegini N (1994) Neem oil inhibits two-cell embryo development and trophectoderm attachment and proliferation *in vitro*. J Assist Reprod Genet 11: 419–427 http://www.springerlink.com/content/7hhx8n434427229u/

Kaldorf M, Koch B, Rexer K-H, Kost G, Varma A (2005) Patterns of interaction between Populus Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biol 7: 210–218

Kassem A, Aghbari AA, Habori AM, Mamary AM (2006) Evaluation of the potential antifertility effect of fenugreek seeds in male and female rabbits. Contraception 73: 301–306

Kumari R, Yadav H K, Bhoon YK, Varma A (2003) Colonization of Cruciferous plants by *Piriformospora indica*. Curr Sci 85: 1672–1674

Kutney PJ, Hewitt GM, Gin L, Piotrowska K, Roberts M, Rettig SJ (1992) Studies with tissue cultures of the Chinese herbal plant, *Tripterygium wilfordii*. Isolation of metabolites of interest in rheumatoid arthritis, immunosuppression, and male contraceptive activity. Can J of Chem 70: 1455–148

Leake JR (1994) The biology of myco-hetrotrophic (saprophytic) plants, New Phytol. 127: 171–216

Lohiya NK, Goyal RB, Jayaprakash D, Ansari AS, Sharma S (1994) Anti-fertility effects of aqueous extracts of *Carica papaya* seeds in male rats. Planta Med 60: 400–404

Malla R, Prasad R, Kumari R, Giang PH, Pokharel U, Oelmüller R, Varma A (2004) Phosphorus solubilizing symbiotic fungus: *Piriformospra indica*. Endocytobiosis Cell Res 15: 579–600

Mathur R, Gupta SK, Singh N, Mathur S, Kochupillai V, Thirumurthy Velpandian (2006) Evaluation of the effect of *Withania somnifera* root extracts on cell cycle and angiogenesis. J Ethnopharmacol 105: 336–34

Mazumder A, Mazumder R (2006) Traditional knowledge, herbal remedies to anti bacterial therapy: its current status in the 21st century. In: Trivedi PC (ed) Medicinal plants: ethnobotanical approach. Agrobios, Jodhpur, pp 50–51

Odesanmi OS, Dawodu AO, Magbagbeola OA (2002) Comparison of Metabolic effects of ethanolic extracts of *Ancistrophyllum secundiflorum* and menstrogen (orthodox contraceptive) on metabolic parameters in pregnant rabbits. Nigerian J Health Biomed Sci 1: 117–120.

Oelmüller R, Peškan-Berghöfer T, Shahollari B, Trebicka A, Sherameti Irena, Varma A (2005) MATH-domain proteins represent a novel protein family in *Arabidopsis thaliana* and at least one member is modified in roots in the course of a plant/microbe interaction. Physiol Plant 124:152–166

Peškan-Berghöfer T, Shahollari B, Giang P.H., Hehl S, Markent C, Blank V, Kost G, Varma A, Oelmueller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represent a novel system to study beneficial plant-microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. Physiol Plant 122: 465–471

Pham GH, Singh An, Malla R, Kumari R, Prasad R, Sachdev M, Rexer KH, Kost G, Luis P, Kaldorf M, Buscot F, Harrmann S, Peskan T, Oelmüller R, Saxena AK, Declerck S, Mittag M, Stabentheiner E, Hehl S, Varma A (2004a) Interaction of *Piriformospora indica* with diverse microorganisms and plants. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer, Heidelberg, pp 237–265

Pham GH, Kumari R, Singh A, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Peskan T, Weiss M, Hampp R, Varma A (2004b) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L,WernerD, Hampp R (eds) Plant surface microbiology. Springer, Heidelberg, pp 593–616

Pinto E, Pina-Vaz C, Salgueiro L, Gonçalves MJ, Costa-de-Oliveira S, Cavaleiro C, Palmeira A, Rodrigues A, Martinez-de-Oliveira J (2006) Antifungal activity of the essential oil of *Thymus pulegioides* on Candida, Aspergillus and dermatophyte species. J Med Microbiol 55: 1367–1373

Prasad R, Garg A P and Varma A (2004) Interaction of medicinal plants with plant growth promoting rhizobacteria and symbiotic fungi. In: Podila G, Varma A (eds) Basic research and applications: mycorrhizae. Microbiology series, vol 1. IK International, India, pp 363–407

Prasad R, Pham GH, Kumari R, Singh A, Yadav V, Sachdev M, Peskan T, Hehl S, Oelmüller R, Varma A (2005) Sebacinaceae: culturable mycorrhiza-like endosymbiotic fungi and their interaction with non-transformed and transformed roots. In: Declerck S (ed) Root organ culture of mycorrhizal fungi. Soil biology series. Springer, Heidelberg, pp 291–312

Prasad R, Malla R, Bhatnagar K, Das A, Kharkwal H, Verma N, Garg AP and Varma A (2007) Beneficial microorganisms: herbal and medicinal plants. In: Chauhan AK, Harsha K, Varma A (eds) Microbes for human life, vol 4. IK International, India, pp 49–72

Rai MK (1994) Herbal medicines in India: retrospect's and prospects. Fitoterapia LXV 6: 483–491

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Electron J Biotechnol 8: 107–11

Rai MK, Singh A, Arya D, Varma A (2001) Positive growth responses of *Withania somnifera* and *Spilanthes calva* were cultivated with *Piriformospora indica* in field. Mycorrhiza 11: 123–128

Rai MK, Varma A, Pandey A K (2004) Antifungal potential of *Spilanthes calva* after inoculation of *Piriformospora indica*. Mycoses 47: 479–481

Ray S, Jay S (2001) Production of withaferin A in shoot cultures of *Withania somnifera*. Planta 67: 432–436

Read DJ (1999) Mycorrhiza: the state of art. In: Varma A, Hock B (eds) Mycorrhiza: structure, function, molecular biology and biotechnology, 2nd edn. Springer, Heidelberg, pp 3–34

Riddle JM (1997) Eve's herb: a history of contraception and abortion in the west. Harvard University Press, Cambridge, Mass.

Sahay N S, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett 181: 297–302

Sahay N S, Varma A (2000) Biological approach towards increasing the survival rates of micropropagated plants. Curr Sci 78: 126–129

Schüβler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1321

Selosse MA, Bauer R, Moyersoen B (2002) Basal hymenomycetes belonging to Sebacinaceae are ectomycorrhizal on temperate deciduous trees. New Phytol 155: 183–195

Serfling A, Wirsel SGR, Lind V, Deising HB (2007) Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field Conditions. Phytopathology 97: 523–531

Shahollari B, Varma A, Oelmüller R (2005) Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162: 945–958

Singh A, Varma A (2000) Orchidaceous Mycorrhizal fungi. In: Mukherji KG (ed) Mycorrhizal fungi. Kluwer, Amsterdam, pp 265–288

Singh An, Singh A, Rexer KH, Kost G, Varma A (2001) Root endosymbiont: *Piriformospora indica* – A Boon for Orchids. J Orc Soc India 15: 89–102

Singh An, Singh Ar, Kumari M, Rai MK, Varma A (2003a) Biotechnology importance of *Piriformospora indica*-A novel symbiotic mycorrhiza-like fungus: an overview. Plant Biotechnol (special issue) 2: 65–75

Singh An, Singh Ar, Kumari M, Kumari R, Rai MK, Sharma AP, Varma A (2003b) AMF-like-fungus: *Piriformospora indica*-a boon for plant industry. In: Prasad BN (ed) Biotechnology in sustainable biodiversity and food security. Oxford and IBH, New Delhi, pp 101–124

Singh Ar, Singh An, Varma A (2002) *Piriformospora indica-in vitro* raised leguminous plants: A new dimension in establishment and phyto-promotion. Indian J Biotech. 1: 372–376

Singh V, Jain DK (1987) Taxonomy of angiosperms, Rastogi, Meerut

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic, London

Siqueira JO, Safir GR, Nair MG (1991) Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytol. 118: 87–93

Tester M, Smith SE, Smith FA (1987) The phenomenon of "nonmycorrhizal" plants. Can J Bot 65: 419–431

Thakur S, Ganpathy PS, Johri BN (1976) Morphogenesis of organ differentiation in *Bacopa monnieri* stem cultures. Indian J Exp Biol 16: 514–516

Tiwari V, Singh BD, Tiwari KN (1998) Shoot regeneration and somatic embryogenesis from different explants of Brahmi (*Bacopa monniera*). Plant Cell Reports 17: 538–543

Urban A, Weiß M, Bauer R (2003) Ectomycorrhizae involving sebacinoid mycobionts. Mycol Res 107: 3–14

Varma A, Rai MK, Sudha N, Sahay N (2000) Microbial-biotechnology: new paradigms and role in sustainable agriculture. In: Rajak RC(ed)Microbial biotechnology for sustainable development and productivity. Science Publishers, India, pp 22–37

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Hurek T, Blechert O, Rexer KH, Kost G, Hahn A, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: an axenically culturable mycorrhiza like endosymbiotic fungus. In: Hock B (ed) The mycota, vol IX. Springer, Heidelberg, pp 125–150

Varma A, Verma S, Sudha, Sahay NS, Butehorn B, Franken P (1999) *Piriformospora indica*, a cultivable plant growth promoting root endophyte. Appl Environ Microbiol 65:2741– 2744

Verma S, Varma A, Rexer KH, Hassel A, Kost G, Sarabhoy A, Bisen P, Bütenhorn B, Franken P (1998) *Piriformospora indica*, gen. et sp. nov., a new root colonizing fungus. Mycologia 90:896–903

Waller W, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt stress tolerance, disease resistance and higher yield. Proc Natl Acad Sci USA 102: 13386–13391

Williamson EM (ed) (2002) Major herbs of Ayurveda. Elsevier, London

Winslow CL, David JK (1998) Herbs as medicines. Arch Intern Med 158: 2192–2199

In vivo Assessment of Stress Impact on Plant's Vitality: Applications in Detecting and Evaluating the Beneficial Role of Mycorrhization on Host Plants

Merope Tsimilli-Michael, Reto J. Strasser(✉)

1 Introduction

Where land exists and the climate is supporting, life develops. Microorganisms, plants, animals and humans populate the site and they together form an ecosystem. Living together is a fact; the life quality of each individual is influenced by each member of the system, and no established life style will persist if the total ecosystem is not in a sustainable state. The maximization of exploitation of the resources by man leads in the long term, in all cases, to a disaster. Only optimization of the use of resources combined with recycling of waste and the regeneration of used areas can lead to sustainability. Every project based on a harmonic balance between the consumption and production of goods with respect towards the environment contributes to *sustainability* (Strasser and Tsimilli-Michael 1998). A tremendous amount of know-how is available and no method that can serve the purpose should be rejected. No problem of major importance can be solved by a single person. We have all to learn and to try how sustainability can be established. Every person is invited to participate in such projects with his knowledge and experience to build up a network of "Collective intelligence".

The work presented here aims to assess the vitality of plants, which is a prerequisite for sustainability and the conservation of biodiversity. Sustainable agriculture is today both a challenge and a necessity. More than a system of farming, it is a new approach integrating ecological, sociological, economical and even cultural aspects of life. The *multidisciplinary character* of this approach not only allows but requests the involvement of scientists from different fields, which will form a

Dedicated to Prof. Dr. Hubert GREPPIN, former Dean of the Faculty of Science and Director of the Department of Plant Biology, University of Geneva, Switzerland, for his steady interest and support to approach Plant Physiology and Bioenergetics with new ways, different than the current trends.

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collective intelligence. It needs a harmonious constellation of political engagement, financial investment and scientific competence to improve life quality in a developing and modern society. Facilitated information-exchange through the Internet links politicians, industrialists and scientists with everybody in a huge net of collective intelligence. *The progress in the democratic concept in this century is based on transparency*.

2 Assessing Plant's Vitality in Terms of Activity and Adaptability: Theoretical Frame and Experimental Procedure

The general axis of our research in the Laboratory of Bioenergetics of the University of Geneva is the description of plant's vitality in terms of activity and adaptability. Hence, our main aims are:

- To develop theories which are able to describe the dynamic behavior of complex systems such as plants and ecosystems.
- To develop experimental procedures which allow to measure and test the samples, so that the theories can be checked.
- To propose and promote the construction of new instruments which allow us to measure and quantify the behavior of plants in their natural environment.

In this framework, the main goals for any project, further than the fundamental curiosity to understand better the complexity of a photosynthetic system in its perpetually changing environment, can be summarized as follows:

- To increase the cross-talk between the sample and the scientist by using different and new experimental approaches; the construction of new types of instruments becomes a part of the project.
- To test different intellectual approaches to "digest" the experimentally available signals and to translate them into biological meanings, such as:
  - modeling to test the possibility of simulation,
  - fitting where simulation is possible,
  - conceptual transformation of fitted experimental parameters into expressions of biophysical and biochemical meanings.

  All these for one set of a sample of one cultivar in a given physiological state.
- To compare and organize, according to similarities and different behavior patterns, a big number of datasets.
- To extend the already existing energy flux theory of photosynthetic membranes for the description of the stability and variability of a system upon environmental changes.
- To target a pragmatic application of the findings in precision agriculture, environmental stress biology, biotechnology and vitality testing for new transgenic cultivars.

2.1 Stress and Stress Adaptation: Theoretical Approach

The theoretical background of our approach is that the vitality of each organism in the ecosystem strongly depends on its adaptability to the certain environmental conditions. We therefore deal with stress and stress adaptation. The term "stress" comes from physics where it has been precisely defined and related to the strain it provokes. However, concerning biology, it has been given widely differing meanings. Probably due to an extension of the physical meaning, many of them converge in attributing stress to any environmental factor "unfavorable" for the living organism under consideration.

Our approach is different in principle. It is focused on the dynamic character of the relationship between organism and environment, keeping from the physical approach the concept of "action-reaction", and offers the possibility of analytical description and quantification. The adaptation of plants to a continuously changing environment is approached as an expression of the general adaptive and evolutionary behavior of organisms, which is an optimization strategy. Based on inferences from open system thermodynamics, this strategy can be regarded as dictated by the thermodynamic demand for minimal entropy production leading to optimal states which are states of stability. When a thermodynamically optimal state is achieved, the system is characterized as being in harmony with its environment. Any environmental change is a stressor in the sense that it disturbs the achieved optimality and leads the system into suboptimality. Stress, defined as the deviation from optimality, has therefore a relative meaning with nonstress as the reference condition. Because of the thermodynamic demand for optimality, suboptimality creates a force under which the system undergoes state changes, i.e. structural/conformational changes, searching for a new optimal state. (For the stress concept see: Strasser 1985, 1988; Strasser and Tsimilli-Michael 1998, 2005; Tsimilli-Michael and Strasser 2002; Tsimilli-Michael et al. 1995, 1996.)

2.2 Biophysical Phenomics: In Vivo Analysis of Plant's Vitality Leads to the Description of their Biophysical Phenotype

The goal of Biophysical Phenomics is to describe, by assessing in vivo the vitality of a plant, its biophysical phenotype, i.e. to establish a functional behavior pattern of the living sample (Strasser and Tsimilli-Michael 2005, Strasser et al. 2007). Definitions and requirements are listed as follows:

2.2.1 Definition of Phenomics

- Here we consider *Biophysical Phenomics*.
- Biophysical Phenomics are Data Banks of *Biophysical Phenotyping*.
- Biophysical Phenotyping refers to *in vivo Vitality Analysis*.

- In vivo Vitality Analysis is based on *in vivo Biospectroscopy*.
- In vivo Biospectroscopy supplies *Images, Spectra and Kinetics*.
- The images, spectra and kinetics are experimental *Absorption-, Reflection-, and Fluorescence-Signals*.
- The experimental signals of the sample are transformed into a *Behavior Pattern*.
- The behavior patterns of the worldwide collected samples are stored in a *Data Bank*.
- This is a *Data Bank of Phenomics*.

2.2.2 Requirements of Biophysical Phenomics

- The measurements have to be conducted in a short time on a big number of samples to allow screening and statistical treatment of the data (a few seconds measuring time per sample).
- The type of samples can vary a lot, such as leaves, microplants, tissue cultures, suspensions of cells or chloroplasts, or colonies of algae on agar plates.
- The instrument has to be lightweight, portable and independent of external power sources for more than one working day.
- The instrument can go to the sample, to the field, in the greenhouse or at the laboratory.
- The original data should be digitized on line in real time and stored in core memory.

2.3 Experimental Procedure

The photosynthetic apparatus, and particularly photosystem (PS) II, is very sensitive and needs to undergo state changes all the time to maintain optimal function under a perpetually changing environment. Fluorescence techniques are useful to monitor in vivo state changes in plants. Our experimental procedure detects and evaluates such changes through their impact on functional photosynthetic properties, by analyzing direct fluorescence kinetics of chlorophyll (Chl) *a* emitted by plants and recorded with a high time resolution from microseconds to seconds; commercial instruments such as PEA, FIM, HandyPEA or PocketPEA (Hansatech Instruments, Kings Lynn Norfolk, UK) have been used (Strasser et al. 2004).

We concentrate on the fast polyphasic fluorescence increase, from 10 µs to 1 s, exhibited by leaves at different physiological states (Strasser and Govindjee 1992; Strasser et al. 1995). This increase shows the clearly visible steps O, J, I and P (hence denoted as OJIP). Using the derivative of a transient, or the differences and/or ratios of suitably normalized transients exhibited by stressed and nonstressed plants, allows the detection of more bands/phases, O-L-K-J-I-H-G-P (Strasser et al. 2004); the bands between F_0 and F_P are labelled in alphabetic order, from slower to faster events. These phases provide a wealth of information concerning the presence and function of mechanisms governing important

functional photosynthetic properties. Moreover, the analysis of the raw fluorescence transients OJIP by the equations of the so called JIP-test, which have been formulated on the basis of the Energy Flux Theory in Biomembranes (Strasser 1978, 1981), leads to a quantification of these properties, hence the evaluation of the impact of the state changes induced by stress. (For the JIP-test and applications in stress detection and evaluation, see: Strasser and Strasser 1995; Srivastava and Strasser 1995, 1996; Srivastava et al. 1997; Krüger et al. 1997; Ouzounidou et al. 1997; Strasser and Tsimilli-Michael 2001; Tsimilli-Michael and Strasser 2001, 2002; Tsimilli-Michael et al. 1995, 1996, 1998, 1999, 2000; Clark et al. 1998, 2000; Prakash et al. 2003; Epitalawage et al. 2003; Hermans et al. 2003; Van Heerden et al. 2003; for reviews, see: Strasser et al. 2000, 2004.)

The JIP-test, which is a well-elaborated procedure, has been developed in the laboratory of Bioenergetics of Geneva and is today used worldwide as it can be easily used for the description of plants under stress and can, thus, serve as a tool for screening the vitality of plants in any biotope.

Data banks can be established with the raw experimental data and with the elaborated data such as:

- Fluorescence images (Fig. 1)
- Fluorescence OJIP kinetics (Fig. 2)
- Carpet plot of many samples (Fig. 3)

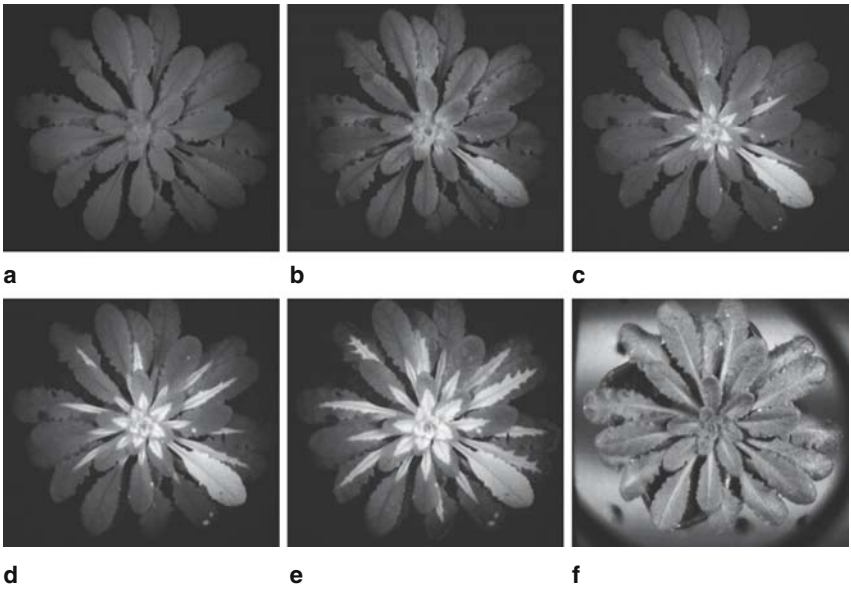

Fig. 1 Chlorophyll *a* fluorescence imaging in *Arabidopsis* before (**a**) and 2 h (**b**), 4 h (**c**), 6 h (**d**), 10 h (**e**) after diuron application; **f** greyscale reflectance image of the treated rosette. Diuron inhibits the photosynthetic electron transport, hence, energy dissipation as heat and fluorescence increases. (From Chaerle et al. 2003, with permission of the publisher)

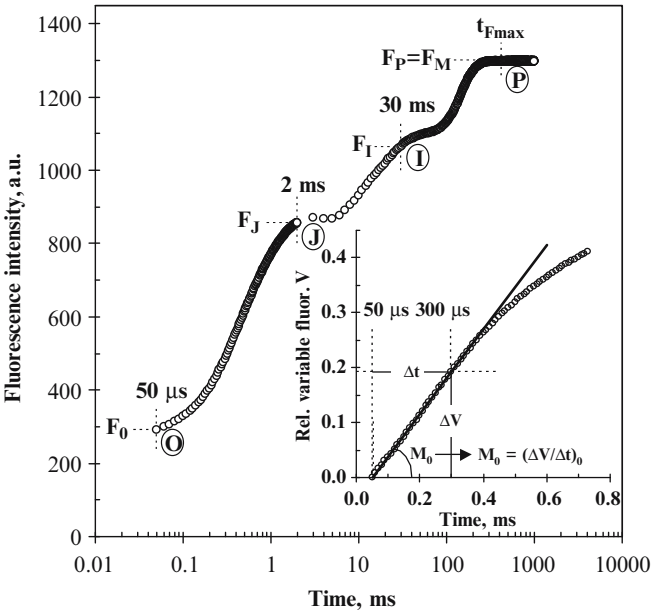

Fig. 2 A typical Chl *a* polyphasic fluorescence rise OJIP, exhibited by higher plants, is plotted on a logarithmic time scale from 50 µs to 1 s. The marks refer to the selected fluorescence data used by the JIP-test for the calculation of structural and functional parameters. The data are: the fluorescence intensities F_0 (at 50 µs), F_J (at 2 ms) and F_I (at 30 ms); the maximal fluorescence intensity, $F_P = F_M$ (at t_{Fmax}). *Insert* The transient is expressed as the relative variable fluorescence $V = (F - F_0)/(F_M - F_0)$ versus time, from 50 µs to 0.8 ms, on a linear time scale, demonstrating also how the initial slope is calculated: $M_0 = (dV/dt)_0 \cong (\Delta V/\Delta t)_0 = (V_{300\mu s})/(0.25\,ms) = 4(F_{300\mu s} - F_0)/(F_M - F_0)$. (From Tsimilli-Michael et al. 2000, with permission of the publisher)

- Radar (spider) plot for parameter pattern recognition (Fig. 4)
- Pipeline plots showing the energy fluxes of different samples (Fig. 5)

The laboratory of Bioenergetics of the University of Geneva answers with pleasure messages from people who are interested in environmental questions. For several years now, experimental data from around the world, that can be analyzed by the JIP-test to describe the vitality of plants, reach our laboratory in Geneva by electronic means. After processing the data, the results are made available, often for teaching purpose, to everybody, everywhere. (BIOLYZER is the software for automatic data processing for calculating the JIP-test parameters. It is available on the net as free software (www.unige.ch/sciences/biologie/bioen/), or directly from the Bioenergetics Laboratory, University of Geneva, Switzerland).

With technology progress, new types of measurements are now available, such as laser induced fluorescence imaging and fluorescence remote sensing by satellite. Our goal is to collaborate in international programs to contribute with our know-how and our experience to the scientific network.

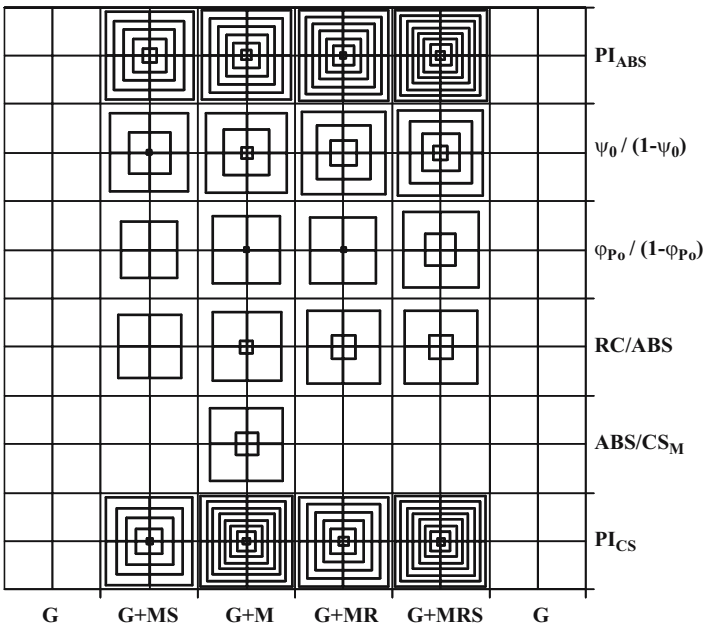

Fig. 3 The effect of single inoculation by AMF (M) and of co-inoculations (MS, MR, MRS) with the diazotroph bacteria Azospirillum (S) and/or Rhizobium (R), all in gamma-sterilised soil (G), on PSII behaviour in alfalfa. PSII behavior is quantified by the performance indexes PI_{ABS} and PI_{CS}, presented together with their components. The values of each parameter are normalized on the corresponding value of G. The logarithms of the normalized values, demonstrated by a so-called "carpet plot", are visualized by the number of the contour lines, which are spaced with an equi-distance of 0.05 (here bigger than the control). (From Tsimilli-Michael et al. 2000, with permission of the publisher)

2.4 Systems Biology and Phenomics

In vivo vitality testing and characterization are procedures to study the dynamics of a whole living system in its environment.

According to open system thermodynamics, the Gibb's energy, linked to biochemical activity (*quantity term*), and the entropy-related energy component, linked to structure, complexity and organization of the system (*quality term*), follow optimization strategies, potentially establishing steady-states under given conditions (*stability term*) (Strasser and Tsimilli-Michael 2005).

Recognizing the great complexity and heterogeneity of the photosynthetic system in nature (Strasser and Tsimilli-Michael 2005), we propose that its apparent state is a heterogeneous macrostate, determined by the statistical distribution of microstates, as listed in the model of Fig. 6: Architecture of PSII and PSI antenna, i.e. size and connectivity among units (grouped/separate), light-harvesting complexes

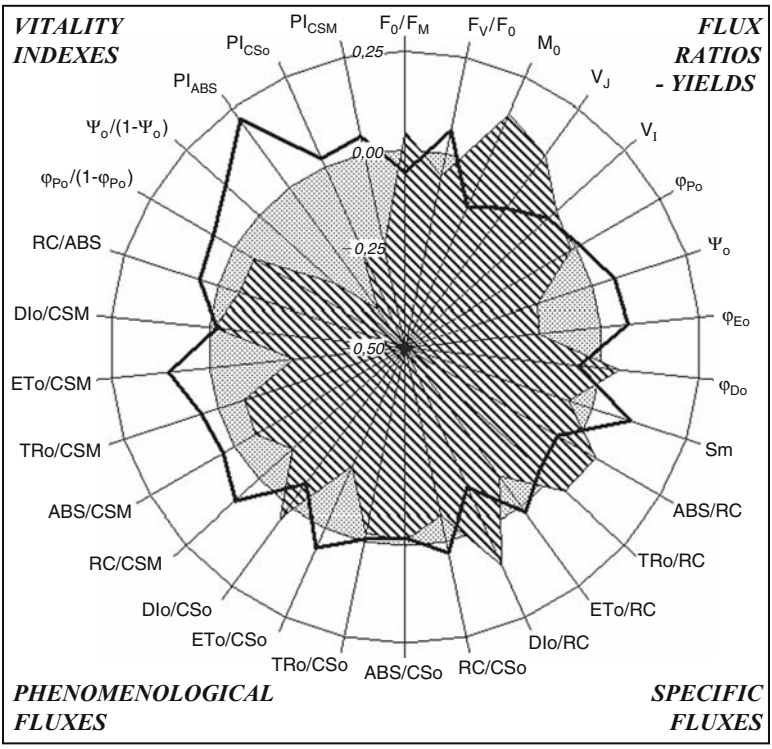

Fig. 4 Radar plot of several parameters of stressed and unstressed trees calculated by the JIP-test and plotted on logarithmic scale (from data of Hermans et al. 2003, with permission of the publisher). For each parameter, the average value of all trees, stressed and nonstressed, is used as the basis for normalization; it is thus presented by the regular *polygon* (around the *grey area*). The *heavy black line* presents the normalized values of the parameters of the control (nonstressed) trees and the *line* enclosing the *hatched area* those of the stressed trees. (For the description of the presented parameters, see text and Strasser et al. 2004)

(LHC II and I), LHC-migration catalyzed by kinase, i.e. part of LHC moving from PSII to PSI when phosphorylated (~P), spill-over from PSII to PSI antenna, types of electron donation to PSII RC (from water oxidation or internal/external donors), Q_A reducing reaction centers (RC) or non Q_A reducing (heat sinks), Q_B reducing or non Q_B reducing (slow Q_A reoxidizing) units, states of intermediate electron carriers (PQ pool, Cytb6/f, PC) and PSI acceptor side, split in non NADP reducing pathways and NADP reducing pathways, the latter further split in non CO_2 fixing and towards CO_2 fixation. For any steady-macrostate (optimal/adapted), the balance of mechanisms governing the distribution of microstates is equivalent to *optimizations of quantity, quality and stability*, which are interrelated, governed by the genetics of the system, its resources and its environment. For specific environmental or genetic conditions (for example, low or high light, low or high nitrogen, with or without chlorophyll *b*, etc.) the photosynthetic samples change the statistical distribution of

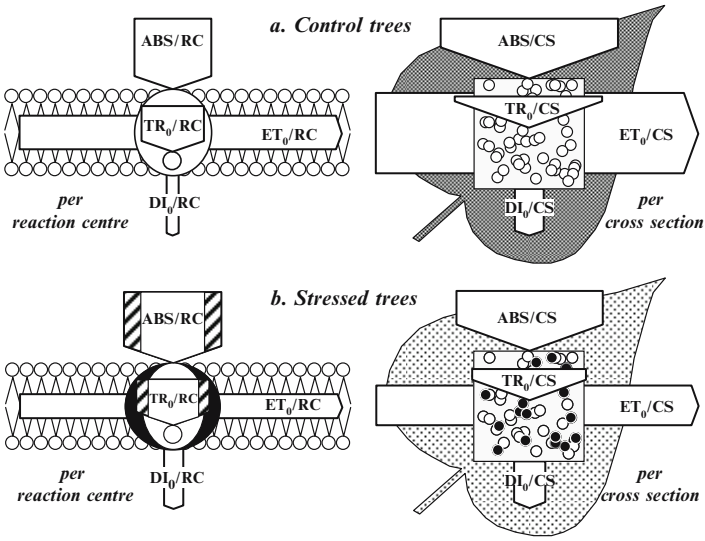

Fig. 5 Energy pipeline models of (*a*) nonstressed (control) and (*b*) stressed trees. The membrane models (*left*) present the specific energy fluxes (per reaction centre, RC). The leaf models (*right*) present the phenomenological energy fluxes (per excited cross section, CS). The relative magnitude of each flux is shown by the width of the corresponding *arrow*. The apparent antenna size follows the value of ABS/RC; it expresses the total absorption flux of PSII antenna chlorophylls, both of active and inactive (so called heat sinks or silent) centres, divided by the number of only the active RCs. In the membrane model, the absorption and trapping by inactive centres are indicated with the *hatched* parts of the corresponding *arrows*; the antenna belonging to PSII units with inactive centres is drawn in *black*. In the leaf model, *open circles* indicate the active RCs and *closed circles* the inactive centres. The darkness of the foliage indicates, qualitatively, the chlorophyll concentration per leaf cross section. (From Hermans et al. 2003, with permission of the publisher)

the possible microstates. In this conceptual frame, stress is any disturbance of the achieved balance, upon which the system undergoes microstate-changes towards a new optimal balance or macrostate (Strasser and Tsimilli-Michael 2005; Strasser et al. 2007).

It is of analytical interest to know the natural dynamic ranges of the heterogeneity of the microstates. At present, we distinguish experimentally with our methods under in vivo conditions the following heterogeneities, all referring to PSII: (1) separate and energetically connected (grouped) units, (2) Q_A and non Q_A reducing units, (3) Q_B and non Q_B reducing centres, and (4) oxygen and non oxygen evolving units; all measurable under physiological and reversible conditions. Extension of the modeling and analytic possibilities to include PSI activity (Strasser et al. 2004) and gas exchange measurements (van Heerden et al. 2003) will increase the number of experimentally distinguishable heterogeneous states and their quantification.

The simulation and fitting of the heterogeneous building blocks will allow the quantification of the statistical distribution of different photosynthetic microstates within the thylakoids; this heterogeneity determines the productivity (e.g. *crop*

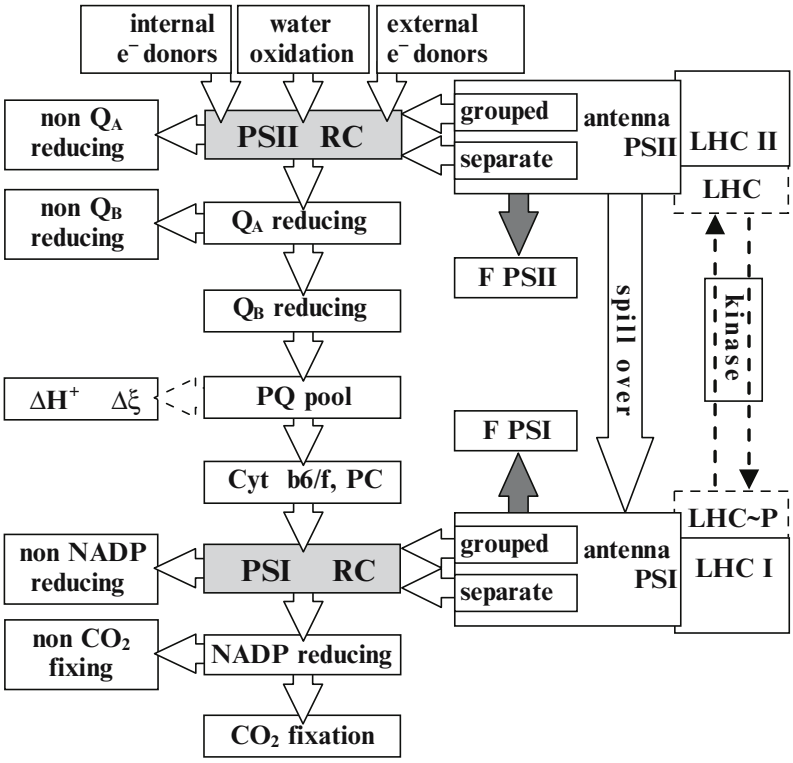

Fig. 6 Heterogeneity of microstates/functional units, whose balance determines the macrostate of the photosynthetic system (modified after Strasser and Tsimilli-Michael 2005)

yield) and stability (e.g. *stress resistance*) of a system. Therefore, the selection of new crops after breeding or genetic manipulations can be done according to functional criteria of biophysical phenomics, e.g. by the analysis of the fast OJIP fluorescence rise with the JIP-test.

3 Case studies: Mycorrhization Effectiveness Probed by the JIP-test

3.1 Beneficial Role of Endo- and Ectomycorrhiza and Piriformospora indica

The beneficial effect of microsymbionts on the health of the host plants has been widely demonstrated. In many cases, they result in an increase in growth and crop yield, especially when the soil conditions (for example, nutrient-deficient degraded habitats, or polluted soil) are suboptimal for the plant or during stress periods (see, e.g.,

Varma 1995, 1998; Varma and Schuepp 1996; Biro et al. 2006). They are therefore a main parameter in ecosystem functions and highly advantageous in sustainable agriculture. In the frame of our stress concept we consider that these symbionts act as *stress-buffers*, since they result in an increased stress resistance, i.e. in the *strengthening* of the plants.

Mycorrhizae (ecto- and endo-) are mutualistic microsymbionts of about 90% of higher plants in natural, semi-natural and agricultural plant communities, with a well-documented beneficial role. However, they are *obligate* symbiotic fungi, i.e. they cannot be cultivated without a higher plant as host.

Piriformospora indica is an emerging growth booster with arbuscular mycorrhizal fungi (AMF)-like characteristics and the added big advantage of being able to grow axenically; it is cultivable in vitro, on agar plates or in liquid cultures (Verma et al. 1998; Varma 1999, 2001). This fungus can colonize all tested higher plants so far, including *Arabidopsis* and conifers, and is therefore a *facultative* root-symbiont. Inoculation with *P. indica* and application of fungal culture filtrate increases the tolerance to temperature and drought, as well as to heavy metals (see references in Strasser et al. 2007). Moreover, *P. indica* has the properties of bioferilizer, bioregulator, phytoremediator, immunomodulator and antioxidants/drugs enhancer (Varma, personal communication).

The use of *P. indica* cultures in precision farming has therefore a big economic potential. However, the success of any microbial inoculation in practise has to be tested for each case, since the effectiveness of symbiosis depends on complex interactions between plant, symbionts and environmental conditions.

3.2 Probing the Effectiveness of Mycorrhization in Applied Projects

Mycorrhizal activity has multiple effects on the physiology and vitality of the host plant. For the detection and evaluation of mycorrhization effectiveness we apply biophysical phenomics; we perform the in vivo vitality analysis of PSII that we use for stress detection and evaluation, as summarized in Section 2 (Tsimilli-Michael et al. 2000; Tsimilli-Michael and Strasser 2002; Biro et al. 2006). The PSII structural and functional parameters assessed by the analysis of the fluorescence transients with the JIP-test provide a quantification of the three (interrelated) components of plant's vitality, namely activity, adaptability and stability.

We here present a pragmatic application of biophysical phenomics of PSII for screening in vivo the impact of symbiosis on the vitality of host plants in two running projects in Cyprus, where typical mycorrhiza (endo- and ecto-) and *P. indica* have been used.

3.3 Experimental Sites, Plants and Inocula for the Case Studies

Two big projects applying mycorrhization are running in Cyprus (2005–2008): reforestation with pine (*Pinus halepensis*) trees (Project: Innovative Biological

Approaches for the Reforestation of Environmentally Stressed Sites - IBARESS II) and vegetable farming (Project: Mycorrhizae for Vegetable Farming). The projects are designed, implemented, funded and coordinated by the United Nations Development Programme (area of work: Environment & Energy for Sustainable Development) in the frame of Action for Cooperation and Trust in Cyprus (UNDP-ACT).

Three case studies are presented here, where the vitality of plants is assessed with the experimental procedure described in Section 2.3. The two refer to parts of the UNDP projects running in Cyprus, namely, reforestation of a quarry in Gypsum with pine (*Pinus halepensis*) trees, and onions (*Allium ceppa*) farming in greenhouse (research facilities in the Turkish Cypriot Community). The pine trees were inoculated with commercial ectomyccorrhizae (AEGIS Ecto Gel - SYTEN Company; composed of a spore mixture of *Rhizopogon* sp., *Pisolithus* sp. and *Schleroderma* sp.) by dipping root balls in the gel. The onions in the presented case study were inoculated with commercial endomycorrhizae (AEGIS Endo Gel - SYTEN Company; composed of *Glomus intraradices* and *G. mosseae*), or with *P. indica*. The third case refers to pine trees (*Pinus halepensis*; taken from the same nursery as for the project in Cyprus) cultivated in pots with poor soil in a greenhouse in the Bioenergetics Laboratory, University of Geneva, after inoculation with *P. indica*. In each case, noninoculated plants of the same age were also studied (control samples).

3.4 Macroscopic Effects of Piriformospora indica and Ectomycorrhiza on Conifers

Pine trees *(Pinus halepensis)* have two types of needles, short and long. The short needles cover always the branches, whereas the 3–5 times longer needles appear only under favorable conditions (in terms of water supply and temperature) that permit the plant to store more assimilates. *Pinus halepensis* plants inoculated with ectomycorrhiza in the field (Gypsum-Cyprus) or with *P. indica* in the greenhouse showed, after 1 year of inoculation, similar macroscopic differences from the corresponding noninoculated plants: a remarkably well recognizable stimulation in growth and 3- to 5-fold higher number of long needles per branch length (Fig. 7). These macroscopic effects indicate already that ectomycorrhiza and *P. indica* are similarly effective in poor soil conditions. However, biophysical evidence is needed for the quantification and the scientific understanding of such symbiotic phenomena.

3.5 Biophysical Phenomics of PSII

Several hundred short and long needles were collected from the pine trees of the reforestation field experiment, 1 year after the trees were planted in the field

Fig. 7 Pine trees (*Pinus halepensis*) grown in pots in a greenhouse (Bioenergetics Laboratory, University of Geneva). The picture was taken 1 year after they were transferred from Cyprus to the pots and the left one was inoculated with *Piriformospora indica*

(Gypsum-Cyprus) and were inoculated (half of them) with a commercial mixture of ectomycorrhizae. For each measurement, a small bundle of 5–10 needles was placed in a leaf clip of a PocketPEA fluorimeter and dark adapted for at least 1 h. An OJIP fluorescence transient induced by a 1 s illumination with saturating red actinic light (600 W m^{-2}, equivalent to about 3,000 μE s^{-1} m^{-2}; peak at 650 nm) was then recorded (for details, see Strasser et al. 1995). The samples were bundles of short needles (S) and long needles (L) from inoculated (M) and noninoculated (C; control) trees. Figure 8 presents, for each of the four cases, the average of the raw fluorescence data from 150 samples. Differences are observed among the four transients, which however can only be evaluated when suitable normalisations and subtractions are performed and/or when the transients are translated by the JIP-test equations to biophysical parameters that can be then quantitatively compared (Section 2.3).

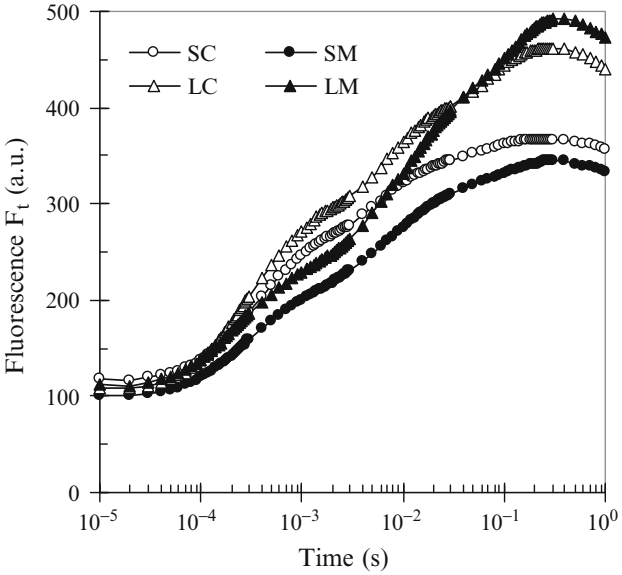

Fig. 8 Chl *a* fluorescence transients of dark adapted (for at least one hour) needles of pine trees peas (*Pinus halepensis*) measured 1 year after they were planted in the field (Gypsum-Cyprus) and inoculated with a commercial mixture of ectomycorrhizae. Each transient presents the average of raw fluorescence data from 150 samples, each consisting of a bundle of 5–10 needles. The samples were short (S) and long (L) needles from inoculated (M) and noninoculated (C; control) trees (hence four combinations). For further details see text

3.5.1 Normalisation and Subtraction of Transients

Figure 9 presents the Chl *a* fluorescence data F_t of Fig. 8 as kinetics of the relative variable fluorescence $V_t = (F_t - F_0)/(F_M - F_0)$ (left panel) and as the difference kinetics ΔV_t (right panel), where F_0 is taken at 20 µs. The ΔV_t kinetics refer to the differences of the V_t of all cases from that of the short needles from noninoculated trees (SC), i.e. LC-SC, SM-SC and LM-SC; an additional ΔV_t kinetics refers to the difference of the long needles of inoculated from the long needles of noninoculated (LM-LC). All difference kinetics are dominated by a negative band, with more pronounced that of the LM-SC difference. The amplitudes of the bands are maximal at the J-step (2 ms; see Fig. 2), thus showing a *higher probability of energy conservation* of the excitation energy trapped by the reaction centre (RC). The band of the LC-SC difference has a different shape, with a maximum at 5–10 ms. Though this is not related to mycorrhization, it is interesting to see that it is also negative at 2 ms, i.e. it also shows an increased probability of energy conservation in the long compared to the short needles of control plants. Furthermore, the maximal amplitude of the LC-SC difference appears at the same time when a secondary band appears in the other differences. This band indicates an *increased capacity* of the PSI electron

acceptor side. The direct impact of mycorrhization is depicted by the SM-SC and LM-LC difference kinetics; it is also worth noting that we can clearly see that these difference kinetics have the same shape, with the difference between long needles having the bigger amplitude. However, we should furthermore consider that at the plant level mycorrhization increases even more the energy conservation, since it also increases both the number of needles per plant and the relative number of long needles.

We applied the same analysis for Chl *a* fluorescence transients (averages of 20 samples) of dark adapted (for at least 1 h) needles of pine trees (*Pinus halepensis*) grown in the greenhouse (Bioenergetics Laboratory, University of Geneva). The measurements were conducted on samples from noninoculated (C; control) and inoculated (Piri) with *P. indica* plants of the same age, 6 months after they were transferred to the greenhouse and inoculation took place (before long needles appeared). The transients are presented in Fig. 10 as kinetics of the relative variable fluorescence $V_t = (F_t - F_0)/(F_M - F_0)$ and as difference kinetics ΔV_t (Piri-C: V_t of inoculated minus the V_t of control). The negative band of ΔV_t has its maximal amplitude at the J-step, showing that, similarly to the case of inoculation with ectomycorrhizae (Fig. 9), *P. indica* results in a *higher probability of energy conservation*. However, in the case of inoculation with *P. indica* (Fig. 10) a second negative band appears, with a maximal amplitude at 200 µs, i.e. in the region of the so-called L-band. According to the Grouping Concept (Strasser 1978, 1981) and the JIP-test analysis (for a review, see Strasser et al. 2004), this observation means that the OJIP transient from the inoculated plants is more sigmoidal than that from the noninoculated, which, in turn, means that an additional impact of

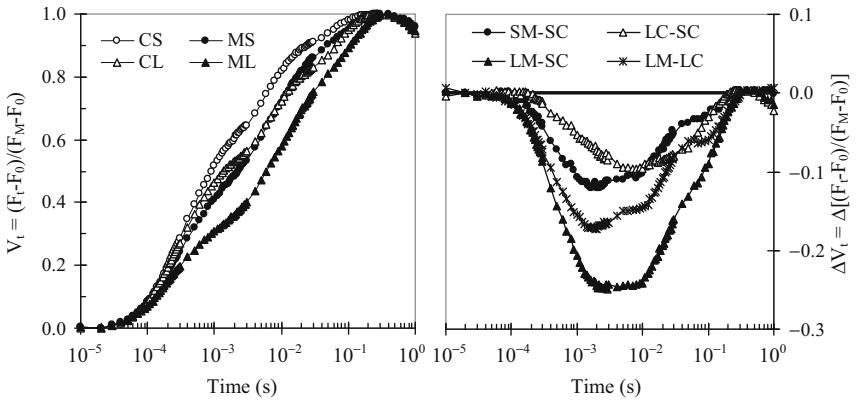

Fig. 9 Presentation of the Chl *a* fluorescence data F_t of Fig. 8 as kinetics of the relative variable fluorescence $V_t = (F_t - F_0)/(F_M - F_0)$ (*left panel*) and as the difference kinetics ΔV_t (*right panel*). The ΔV_t kinetics refer to the differences of the V_t of all cases from that of the short needles from noninoculated trees (SC), i.e. LC-SC, SM-SC and LM-SC; an additional ΔV_t kinetics refers to the difference of the long needles of inoculated from the long needles of noninoculated (LM-LC), while the *heavy black line* marks the zero level of ΔV_t (i.e. the trivially equal to zero differences SC-SC and LC-LC). For further details see text

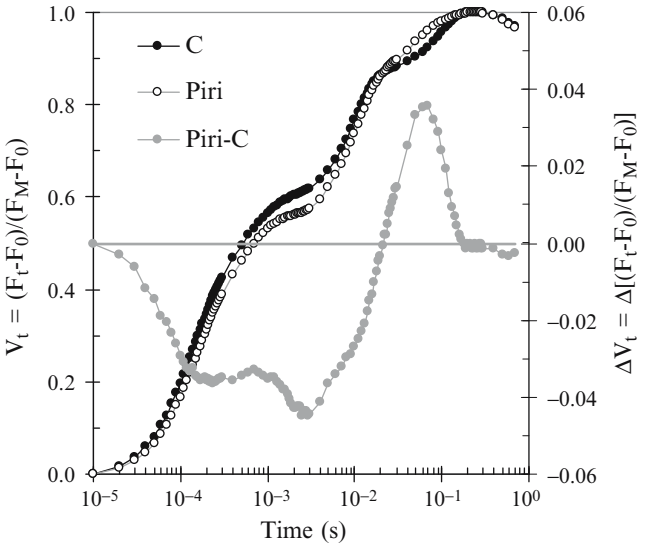

Fig. 10 Chl *a* fluorescence transients (averages of 20 samples) of dark adapted (for at least 1 h) needles of pine trees (*Pinus halepensis*) grown in a greenhouse (Bioenergetics Laboratory, University of Geneva). The measurements were conducted on samples from noninoculated (C; control) and inoculated (Piri) with *Piriformospora indica* plants of the same age, 6 months after they were transferred to the greenhouse and inoculation took place (before long needles appeared). The transients are presented as kinetics of the relative variable fluorescence $V_t = (F_t - F_0)/ (F_M - F_0)$ (*open* and *closed black circles, left axis*) and as difference kinetics ΔV_t (Piri-C: V_t of inoculated minus the V_t of control; *grey closed symbols, right axis*); the *heavy grey line* marks the zero level of ΔV_t (i.e. the trivially equal to zero difference C-C). For further details see text

P. indica was the increase of energetic grouping or cooperativity between PSII photosynthetic units. The physiological effect of a higher grouping probability is that it contributes to a higher *stability* of the sample towards stress conditions. The difference kinetics shows also a positive band in the I-P region (30 ms to about 300 ms), which indicates that inoculation results in a faster fractional reduction of the PSI electron acceptors.

3.5.2 Behavior Patterns - PSII Biophysical Parameters Derived by the JIP-test Equations

The fluorescence transients were analysed according to the JIP-test equations. Each raw transient was analysed and the calculated biophysical parameters of PSII were averaged for each case.

A schematic summary of the JIP-test is shown in Fig. 11, presenting the energy fluxes and their bifurcations, as well as the efficiencies/yields, which are defined as ratios of the energy fluxes.

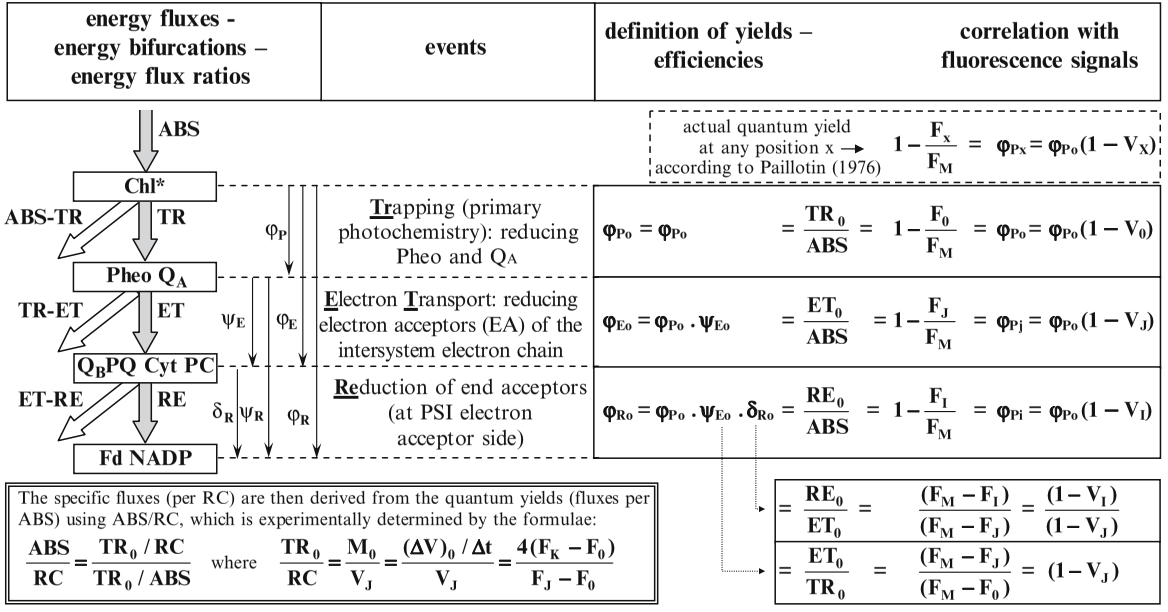
energy fluxes - energy bifurcations – energy flux ratios
events
definition of yields – efficiencies
correlation with fluorescence signals
ABS
Chl*
ABS-TR
TR
Pheo Q_A
TR-ET
ET
Q_BPQ Cyt PC
ET-RE
RE
Fd NADP
φ_P
ψ_E
φ_E
δ_R
ψ_R
φ_R
Trapping (primary photochemistry): reducing Pheo and Q_A
Electron Transport: reducing electron acceptors (EA) of the intersystem electron chain
Reduction of end acceptors (at PSI electron acceptor side)
actual quantum yield at any position x → according to Paillotin (1976) $1-\frac{F_x}{F_M} = \varphi_{Px} = \varphi_{Po}(1-V_X)$
$\varphi_{Po} = \varphi_{Po} = \frac{TR_0}{ABS} = 1-\frac{F_0}{F_M} = \varphi_{Po} = \varphi_{Po}(1-V_0)$
$\varphi_{Eo} = \varphi_{Po} \cdot \psi_{Eo} = \frac{ET_0}{ABS} = 1-\frac{F_J}{F_M} = \varphi_{Pj} = \varphi_{Po}(1-V_J)$
$\varphi_{Ro} = \varphi_{Po} \cdot \psi_{Eo} \cdot \delta_{Ro} = \frac{RE_0}{ABS} = 1-\frac{F_I}{F_M} = \varphi_{Pi} = \varphi_{Po}(1-V_I)$
The specific fluxes (per RC) are then derived from the quantum yields (fluxes per ABS) using ABS/RC, which is experimentally determined by the formulae:
$\frac{ABS}{RC} = \frac{TR_0/RC}{TR_0/ABS}$ where $\frac{TR_0}{RC} = \frac{M_0}{V_J} = \frac{(\Delta V)_0/\Delta t}{V_J} = \frac{4(F_K-F_0)}{F_J-F_0}$
$= \frac{RE_0}{ET_0} = \frac{(F_M-F_I)}{(F_M-F_J)} = \frac{(1-V_I)}{(1-V_J)}$
$= \frac{ET_0}{TR_0} = \frac{(F_M-F_J)}{(F_M-F_0)} = (1-V_J)$

Fig. 11 A schematic summary of the JIP-test: energy fluxes (*wide arrows*) and energy bifurcations, distinguishing the outfluxes for energy conservation (*grey arrows*) from the outfluxes for storage (e.g. dQ_A^-/dt) or dissipation (*white arrows*) are demonstrated. The efficiencies/yields (*line arrows*), which are defined as ratios of the energy fluxes are also shown and further linked with fluorescence signals selected from the OJIP fluorescence transient. The efficiencies refer to the starting conditions (all RCs open; subscript "0") of the photosynthetic sample at a defined physiological state (e.g. dark-adapted). The derivation of the specific fluxes (fluxes per RC; at the starting conditions) from the quantum yields (efficiencies on absorption basis; otherwise, fluxes per ABS) is also indicated. The fluxes refer to absorption (ABS), trapping (TR_0), i.e. reduction of Pheo and Q_A, electron transport (ET_0) from Q_A^- to the intersystem electron acceptors and reduction of end acceptors at the PSI electron acceptor side (RE_0). For further details, see text

The energy fluxes are: for absorption (ABS); trapping (TR_0), i.e. reduction of Pheo (pheophytin) and Q_A (primary electron quinone acceptor); electron transport (ET_0) from Q_A^- to the intersystem electron acceptors: Q_B (secondary electron quinone acceptor), PQ (plastoquinone), Cyt (cytochrome b_6/f) and PC (plastocyanin); reduction (RE_0) of end acceptors at the PSI electron acceptor side: NADP (nicotinamide adenine dinucleotide phosphate) and Fd (ferredoxin).

The efficiencies/yields are: the maximum quantum yield of primary photochemistry ($TR_0/ABS = \varphi_{Po} = 1-F_0/F_M$); the efficiency ($ET_0/TR_0 = \psi_{Eo} = 1-V_J$) with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the intersystem electron acceptors; the quantum yield of electron transport from Q_A^- to the intersystem electron acceptors ($ET_0/ABS = \varphi_{Eo} = \varphi_{Po} \cdot \psi_{Eo}$); the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PSI end electron acceptors ($RE_0/ET_0 = \delta_{Ro} = (1-V_I)/(1-V_J)$); the quantum yield of electron transport from Q_A^- to the PSI end electron acceptors ($RE_0/ABS = \varphi_{Ro} = \varphi_{Po} . \psi_{Eo} . \delta_{Ro}$); the efficiency with which a trapped exciton can move an electron into the electron transport chain from Q_A^- to the PSI end electron acceptors ($RE_0/TR_0 = \psi_{Ro} = \psi_{Eo} . \delta_{Ro}$).

The efficiencies and energy fluxes refer to the starting conditions (all RCs open; subscript "0") of the photosynthetic sample at a defined physiological state (e.g. dark-adapted). The derivation of the specific fluxes (fluxes per RC; at the starting conditions) from the quantum yields (efficiencies on absorption basis; otherwise, fluxes per ABS) is also depicted.

Figure 11 also demonstrates how the efficiencies/yields are linked with fluorescence signals selected from the OJIP fluorescence transient as follows: F_0, the initial fluorescence, at 20 or 50 µs (with the PEA or the Handy PEA fluorimeter respectively); F_K, at 270 or 300 µs; F_J, at 2 ms;, F_I, at 30 ms; F_M, the maximum fluorescence intensity of the OJIP transient. The initial slope M_0 is calculated for Δt = 0.25 ms (see Fig. 2), i.e. between 20 and 270 µs, or 50 and 300 µs.

Further than the efficiencies and the specific fluxes, the JIP-test calculates also the photosynthetic Performance Index (PI_{ABS}), which is a combined measure of three partial performances, namely those related with the amount of photosynthetic reaction centres (RC/ABS), the maximal energy flux which reaches the PSII reaction centre (TR_0), and the electron transport at the onset of illumination (ET_0):

$$PI_{ABS} = \frac{RC}{ABS} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_{Eo}}{1-\psi_{Eo}} = \frac{RC}{ABS} \cdot \frac{TR_0}{ABS - TR_0} \cdot \frac{ET_0}{TR_0 - ET_0}$$

$$= \frac{1-(F_0/F_M)}{4(F_{300\mu s}-F_0)/(F_J-F_0)} \cdot \frac{(F_M-F_0)}{F_0} \cdot \frac{(F_M-F_J)}{(F_J-F_0)}$$

It should be noted that Fig. 11 contains an extension of the JIP-test, presented here for the first time. This extension utilizes the F_I (at 30 ms) for the definition and calculation of the energy flux RE_0 and the related efficiencies φ_{Ro}, δ_{Ro} and ψ_{Ro}.

Therefore, the total performance index (PI_{total}), measuring the performance up to the PSI end electron acceptors, is defined as:

$$PI_{total} = \frac{RC}{ABS} \cdot \frac{\varphi_{Po}}{1-\varphi_{Po}} \cdot \frac{\psi_{Eo}}{1-\psi_{Eo}} \cdot \frac{\delta_{Ro}}{1-\delta_{Ro}}$$

$$= \frac{RC}{ABS} \cdot \frac{TR_0}{ABS-TR_0} \cdot \frac{ET_0}{TR_0-ET_0} \cdot \frac{RE_0}{ET_0-RE_0}$$

Figure 12 depicts the photosynthetic efficiencies $\varphi_{Po} = TR_0/ABS$, $\psi_{Eo} = ET_0/TR_0$ and $\varphi_{Eo} = ET_0/ABS$ and the Performance Index (PI_{ABS}) of short (S) and long (L) needles from noninoculated (C; control) and inoculated with a commercial mixture of ectomycorrhizae (M) pine trees (*Pinus halepensis*) from the reforestation project in Gypsum-Cyprus. As shown in Fig. 12, mycorrhization results in an *increase of all the photosynthetic efficiencies*; the efficiency of electron transfer ψ_{Eo} is much more affected than φ_{Po}, the efficiency/yield of trapping/primary photochemistry. The reader should notice (Fig. 11) that $\psi_{Eo} = 1\text{-}V_J$, which means that a low J-level results in high ψ_{Eo}; therefore, the information from Fig. 11 is the quantified translation of the information from ΔV_t in Fig. 9. Figure 12 demonstrates also that mycorrhization *markedly enhances the photosynthetic performance index* (PI_{ABS}).

From the case study of pine trees (*Pinus halepensis*) grown with and without *P. indica* in the greenhouse of the Bioenergetics Laboratory, University of Geneva, we present in Fig. 13 a representative behavior pattern of PSII, with eight biophysical parameters: PI_{ABS}, the efficiencies (fluxes ratios) TR_0/ABS, ET_0/ABS, ET_0/TR_0 and

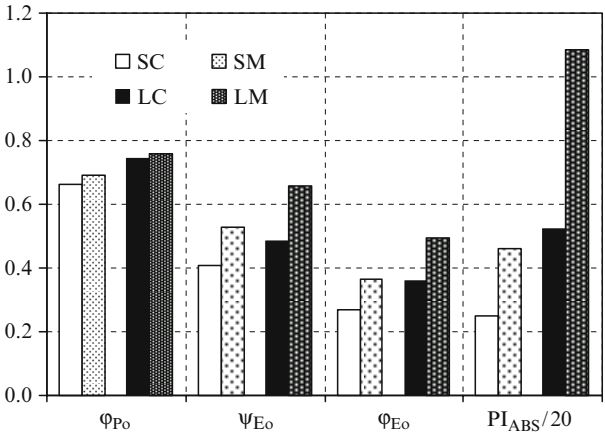

Fig. 12 Photosynthetic efficiencies, φ_{Po}, ψ_{Eo} and φ_{Eo}, and the Performance Index PI_{ABS} (divided by 20 for scaling reasons) of short (S) and long (L) needles from noninoculated (C; control) and inoculated with a commercial mixture of ectomycorrhizae (M) pine trees (*Pinus halepensis*), planted in the field (in Gypsum-Cyprus) (averages of 150 samples). For further details see text

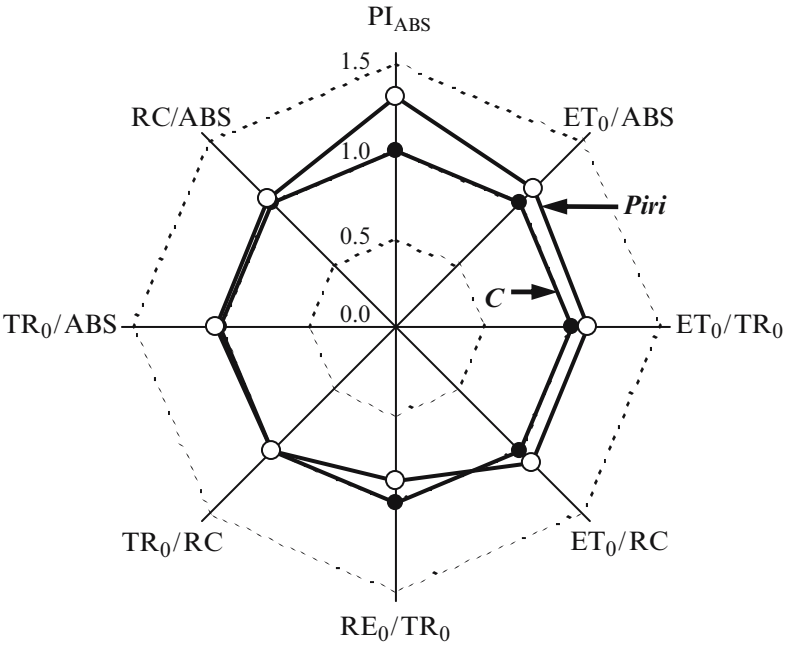

Fig. 13 The impact of symbiosis with *Piriformospora indica* on a behavior pattern of PSII (see Fig. 4) of pine trees (*Pinus halepensis*) grown in a greenhouse (Bioenergetics Laboratory, University of Geneva). The presented behavior patterns (*octagons*) refer to eight structural and functional parameters of PSII, derived by the JIP-test from the fluorescence transients exhibited by needles of inoculated (*Piri*; *open circles*) and noninoculated (*C*; *black circles*) trees of the same age. The parameters are averages of 20 samples from each case. The measurements were conducted 6 months after the plants were transferred to the greenhouse and inoculation took place (before long needles appeared; see Fig. 7). In the plot, each parameter was normalized on that of the control; hence the deviation of the behavior pattern of the inoculated sample from that of the noninoculated (*regular octagon*) demonstrates the fractional impact of symbiosis with *Piriformospora indica*. For further details see text

RE_0/TR_0, the specific fluxes for trapping TR_0/RC and electron transport ET_0/RC, and the amount of photosynthetic reaction centers per absorption RC/ABS. In the plot of Fig. 13, each parameter was normalized on that of the control; hence the deviation of the behavior pattern of the inoculated sample from that of the noninoculated (regular octagon) demonstrates the fractional impact of symbiosis with *P. indica*.

The results from the two case studies of *Pinus halepensis* (depicted in Figs. 9, 10, 12 and 13), demonstrate clearly that *ectomycorrhizae and P. indica exert similar basic effects on the plant's vitality*.

As shown in Fig. 14 for the case of onion (*Allium ceppa*) farming, the similarities of the effects of endomycorrhizae (Myc) and *P. indica* (Piri) on the plant's vitality are extended to all tested parameters; a possible reason is that all tested plants were grown

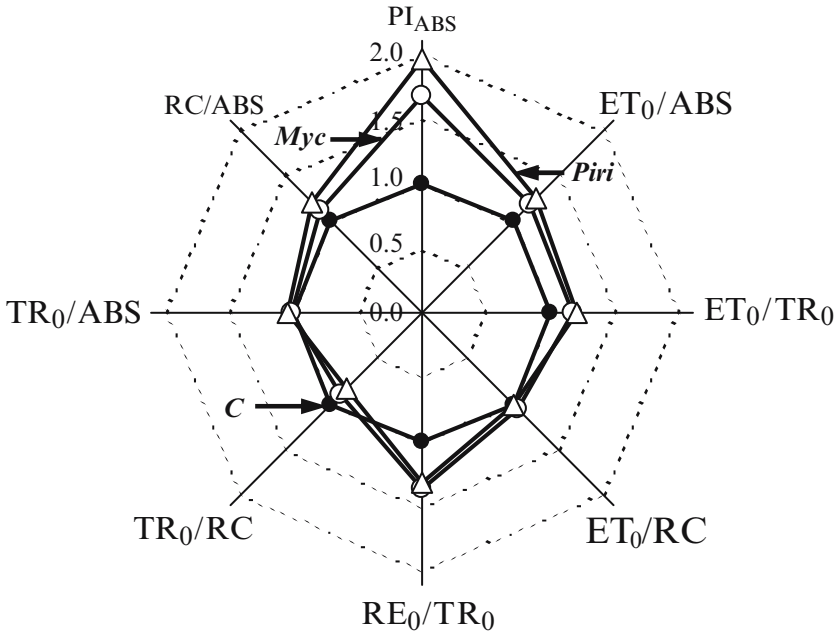

Fig. 14 The impact of mycorrhization on a behavior pattern of PSII (as in Fig. 13) of onions (*Allium ceppa*) grown in a greenhouse (research facilities in the Turkish Cypriot Community). The presented behavior patterns (*octagons*) refer to eight structural and functional parameters of PSII, derived by the JIP-test from the fluorescence transients exhibited by leaves of noninoculated (*C*; *closed circles*) plants and plants of the same age inoculated with a commercial mixture of endomycorrhizae (*Myc*; *open circles*) or *Piriformospora indica* (*Piri*; *open triangles*). The parameters are the averages of 20 samples for each case. In the plot, each parameter was normalized on that of the control; hence the deviations of the behavior patterns of the inoculated samples from that of the noninoculated (*regular octagon*) demonstrate the fractional impacts of symbiosis with endomycorrhiza and with *Piriformospora indica*. For further details see text

in the same environment. For the experiments, three to four onions were planted per pot, with or without inoculation. One day before harvesting the onions, the semi-dried leaf parts were cut away. All leaves are rolled together at the base and become white. About 3 cm above the white limit an OJIP transient was measured in the green zone. For each treatment, at least 50 transients were measured and analyzed separately; the calculated biophysical parameters of PSII were averaged for each case. The behavior patterns depicted in Fig. 14 refer to the same eight structural and functional parameters of PSII as those in Fig. 13 and, similarly, each parameter was normalized on that of the control (C). As clearly shown by the extent of the deviations of the behaviour pattern from the regular octagon corresponding to the control, *the impact of inoculation with P. indica is greater than that of the inoculation with endomycorrhizae*.

It is also interesting to note that symbiosis enhances the Photosynthetic Performance (PI_{ABS}) both in pine trees and onions, though at different extent. It should also be emphasised that the often used parameter F_V/F_M, which stands for the maximum quantum yield of primary photochemistry $\varphi_{Po} = TR_0/ABS$ is highly insensitive to the influence

of the root symbionts, as was also reported before (see, e.g., Strasser et al. 2007) for many cases of stress effects and stress alleviation.

4 Conclusions

The results from the presented three case studies show clearly that increased activity, increased efficiency of energy conservation and increased stress stability are the results of root symbiosis with both typical mycorrhizae and *Piriformospora indica*.

From the experimental point of view, these results demonstrate that biophysical phenomics, which are based on the analysis of the plant's vitality with the JIP-test, provide a powerful tool for the in vivo and in situ recognition and evaluation of the effectiveness of symbiosis, which can not be foreseen or taken for granted as it depends on complex interactions between plant, symbionts and environmental conditions. Our results show, once more, that the Photosynthetic Performance Index PI_{ABS} is the most sensitive parameter used so far, while the commonly used φ_{Po} is highly insensitive even when macroscopic effects can be already recognised.

In summary, the applied techniques have the following advantages:

- they provide an early diagnosis of vitality changes
- they can be used to screen not only leaves but any green part of the plant
- they are rapid – less than a few seconds are needed for each measurement
- they can be applied in vivo and in situ
- they can be carried out anywhere - in the field, in the green house or even in tissue cultures - and even on samples as small as 2 mm^2
- they are not invasive
- they are inexpensive.

It should also be emphasized that, further than the technical/experimental advantages of the applied techniques, all the expressions of the biophysical parameters used are derived on a solid experimental basis (for reviews, see Strasser et al. 2000, 2004).

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References

Biro B, Köves-Péchy K, Tsimilli-Michael M, Strasser RJ (2006) Role of beneficial microsymbionts on the plant performance and plant fitness. In: Mukerji KG, Manoharachary C, Singh J (eds), Microbial activity in the rhizosphere. Soil biology series, vol 7. Springer, Heidelberg, pp 265–296

Chaerle L, Hulsen K, Hermans C, Strasser RJ, Valcke R, Höfte M, Van Der Straeten D (2003) Robotized tile-lapse imaging to assess in-planta uptake of phenylurea herbicides and their microbial degradation. Physiol Plant 118:613–619

Clark AJ, Landolt W, Bucher J, Strasser RJ (1998) The response of *Fagus sylvatica* to elevated CO_2 and ozone probed by the JIP-test based on the chlorophyll fluorescence rise OJIP. In: De Kok LJ, Stulen I (eds) Responses of plant metabolism to air pollution and global change. Backhuys, Leiden, pp 283–286

Clark AJ, Landolt W, Bucher J, Strasser RJ (2000) Beech (*Fagus sylvatica*) response to ozone exposure assessed with a chlorophyll *a* fluorescence performance index. Environ Pollut 109:501–507

Epitalawage N, Eggenberg P, Strasser RJ (2003) Use of fast chlorophyll a fluorescence technique in detecting drought and salinity tolerant chickpea (*Cicer arietinum* L.) varieties. Archs Sci Genève 56:79–93

Hermans C, Smeyers M, Maldonado R, Eyletters M, Strasser RJ, Delhaye J-P (2003) Quality assessment of urban trees: a comparative study of physiological characterisation, airborne imaging and on site fluorescence monitoring by the OJIP-test. J Plant Physiol 160:81–90

Krüger GHJ, Tsimilli-Michael M, Strasser RJ (1997) Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves. Physiol Plantarum 101:265–277

Ouzounidou G, Moustakas M, Strasser RJ (1997) Sites of action of copper in the photosynthetic apparatus of maize leaves: kinetic analysis of chlorophyll fluorescence, oxygen evolution, absorption changes and thermal dissipation as monitored by photoacoustic signals. Aust J Plant Physiol 24:81–90

Paillotin G (1976) Movement of excitations in the photosynthesis domain of photosystem II. J Theor Biol 58:237–252

Prakash JSS, Srivastava A, Strasser RJ, Mohanty P (2003) Senescence-induced alterations in the photosystem II functions of Cucumis sativus cotyledons: probing of senescence driven alterations of photosystem II by chlorophyll *a* fluorescence induction O-J-I-P transients. Indian J Biochem Biophys 40:160–168

Srivastava A, Strasser RJ (1995) How do land plants respond to stress temperature and stress light? Archs Sci Genève 48:135–145

Srivastava A, Strasser RJ (1996) Stress and stress management of land plants during a regular day. J Plant Physiol 148:445–455

Srivastava A, Guisse B, Greppin H, Strasser RJ (1997) Regulation of antenna structure and electron transport in PS II of *Pisum sativum* under elevated temperature probed by the fast polyphasic chlorophyll *a* fluorescence transient OKJIP. Biochim Biophys Acta 1320:95–106

Strasser BJ, Strasser RJ (1995) Measuring fast fluorescence transients to address environmental questions: the JIP-test. In: Mathis P (ed) Photosynthesis: from light to biosphere, vol V. Kluwer, Dordrecht, pp 977–980

Strasser RJ (1978) The grouping model of plant photosynthesis. In: Akoyunoglou G (ed) Chloroplast development. Elsevier, Dordrecht, pp 513–524

Strasser RJ (1981) The grouping model of plant photosynthesis: heterogeneity of photosynthetic units in thylakoids. In: Akoyunoglou G (ed) Photosynthesis III. Structure and molecular organisation of the photosynthetic apparatus. Balaban International, Philadelphia, pp 727–737

Strasser RJ (1985) Dissipative Strukturen als Thermodynamischer Regelkreis des Photosyntheseapparates. Ber Dtsche Bot Ges Bd 98:53–72

Strasser RJ (1988) A concept for stress and its application in remote sensing. In: Lichtenthaler HK (ed) Applications of chlorophyll fluorescence. Kluwer, Dordrecht, pp 333–337

Strasser RJ, Govindjee (1992) The F_0 and the O-J-I-P fluorescence rise in higher plants and algae. In: Argyroudi-Akoyunoglou JH (ed) Regulation of chloroplast biogenesis. Plenum, New York, pp 423–426

Strasser RJ, Tsimilli-Michael M (1998) Sustainability as an optimisation strategy: an application for screening the vitality of a biotope. In: Behl RK, Singh DP and Lodhi GP (eds) Crop improvement for stress tolerance. Ankush, Hisar, India, pp 1–17

Strasser RJ, Tsimilli-Michael M (2001) Stress in plants, from daily rhythm to global changes, detected and quantified by the JIP-test. Chim Nouv 75:3321–3326

Strasser RJ, Tsimilli-Michael M (2005) State-changes realising adaptation to stress as the result of an optimized redistribution of functional microstates. In: van Est A, Bruce D (eds) Photosynthesis: fundamental aspects to global perspectives. Allen Press, Lawrence, Kan., pp 537–540

Strasser RJ, Srivastava A, Govindjee (1995) Polyphasic chlorophyll *a* fluorescence transient in plants and cyanobacteria. Photochem Photobiol 61:32–42

Strasser RJ, Srivastava A, Tsimilli-Michael M (2000) The fluorescence transient as a tool to characterize and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanism, regulation and adaptation. Taylor and Francis, London, pp 443–480

Strasser RJ, Tsimilli-Michael M, Srivastava A (2004) Analysis of the chlorophyll *a* fluorescence transient. In: Papageorgiou GC, Govindjee (eds) Chlorophyll fluorescence: a signature of photosynthesis. Advances in photosynthesis and respiration series, vol 19. Kluwer, Rotterdam, pp 321–362

Strasser RJ, Tsimilli-Michael M, Dangre D, Rai M (2007) Biophysical phenomics reveals functional building blocks of plants systems biology: a case study for the evaluation of the impact of mycorrhization with *Piriformospora indica*. In: Varma A, Oelmüler R (eds), Advanced techniques in soil microbiology. Soil biology series, vol 11. Springer, Heidelberg, pp 319–341

Tsimilli-Michael M, Strasser RJ (2001) Fingerprints for climate changes on the behaviour of the photosynthetic apparatus, monitored by the JIP-test. A case study on light and heat stress adaptation of the symbionts of temperate and coral reef foraminifers in hospite. In: Walther G-R, Burga CA, Edwards PJ (eds) "Fingerprints" of climate changes – adapted behaviour and shifting species ranges. Kluwer, New York, pp 229–247

Tsimilli-Michael M, Strasser RJ (2002) Mycorrhization as a stress adaptation procedure. In: Gianinazzi S, Haselwandter K, Schüepp H, Barea JM (eds) Mycorrhiza technology in agriculture: from genes to bioproducts. Birkhauser, Basel, pp 199–209

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1995) Suboptimality as driving force for adaptation: a study about the correlation of excitation light intensity and the dynamics of fluorescence emission in plants. In: Mathis P (ed) Photosynthesis: from light to biosphere, vol V. Kluwer, Dordrecht, pp 981–984

Tsimilli-Michael M, Krüger GHJ, Strasser RJ (1996) About the perpetual state changes in plants approaching harmony with their environment. Archs Sci Genève 49:173–203

Tsimilli-Michael M, Pêcheux M, Strasser RJ (1998) Vitality and stress adaptation of the symbionts of coral reef and temperate foraminifers probed in hospite by the fluorescence kinetics O-J-I-P. Archs Sci Genève 51:1–36

Tsimilli-Michael M, Pêcheux M, Strasser RJ (1999) Light and heat stress adaptation of the symbionts of temperate and coral reef foraminifers probed in hospite by the chlorophyll a fluorescence kinetics O-J-I-P. Z Naturforsch 54C:671–680

Tsimilli-Michael M, Eggenberg P, Biro B, Köves-Pechy K, Vörös I, Strasser RJ (2000) Synergistic and antagonistic effects of arbuscular mycorrhizal fungi and *Azospirillum* and *Rhizobium*

nitrogen-fixers on the photosynthetic activity of alfalfa, probed by the chlorophyll *a* polyphasic fluorescence transient O-J-I-P. Appl Soil Ecol 15:169–182

Van Heerden PDR., Tsimilli-Michael M, Krüger GHJ, Strasser RJ (2003) Dark chilling effects on soybean genotypes during vegetative development: parallel studies of CO_2 assimilation, chlorophyll *a* fluorescence kinetics O-J-I-P and nitrogen fixation. Physiol Plant 117:476–491

Varma A (1995) Mycorrhizae: their application in micropropagated plantlets. Crit Rev Biotechnol 15:179–199

Varma A (1998) Mycorrhizae, the friendly fungi: what we know, what we should know and how do we know? In: Varma A (ed) Mycorrhiza manual. Springer, Heidelberg, pp. 1–24

Varma A, Schuepp H (1996) Influence of mycorrhization on the growth of micropropagated plants. In: Mukerjee KG (ed) Concepts in mycorrhizal research. Kluwer, Dordrecht, pp 113–132

Varma A, Verma S, Sudha, Sahay N, Britta B, Franken P (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Environ Microbiol 65:2741–2744

Varma A, Singh A, Sudha, Sahay NS, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Varma A, Hock B (eds) Mycota, vol IX. Springer, Heidelberg, pp 123–150

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Bütehorn P, Franken P (1998) *Piriformospora indica* gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:895–909

Edible Mycorrhizal Fungi: Identification, Life Cycle and Morphogenesis

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1 Introduction

In mid-2005, 6.5 billion people shared the planet and, according to 2004 estimates by the population division of the UN Secretariat, world population is expected to rise in the next 45 years by 2.6 billion, to reach a total of 9.1 billion in 2050 (www.unfpa.org). Consequently, one of the main challenges of the twenty-first century will be to produce sufficient food. As illustrated by Morrissey et al. (2004), microbes are important since "Applying knowledge about beneficial plant-microbe interactions […] may allow us to increase food production". Among these microbes, fungi could not only improve plant production, but with about 2,500 edible species recorded, they could also represent an important integrative source of food and incomes (Bao 2004; Wang and Hall 2004). We know that edible fungi represent since long time an appreciated source of food for human populations. The archaeological record reveals edible species associated with people living 13,000 years ago in Chile (Rojas and Mansur 1995). In China, the eating of wild fungi is reliably noted from 6,000–7,000 years ago (Wang 1987). Edible fungi were also collected from forests in ancient Greek and Roman times and highly valued. For instance, we know that truffles were appreciated during antiquity (Callot 1999). Now, they are collected, consumed and sold in over 85 countries worldwide.

In addition to being a source of food, edible fungi are an important source of income for many countries, such as Zimbabwe, Turkey, Poland, China, etc. (Bao 2004) The export trade is driven by a strong and expanding demand from Europe and Japan and is predominantly from poor to rich countries, providing important cash income for local businesses and collectors. Edible fungi are important wildlife food, too (Fogel and Trappe 1978; Maser et al. 1978; Johnson 1996): many mammals feed on fungal fruit bodies (i.e., they are consumed by deer, elks, bears, squirrels, marsupials, primates, etc.). Fungi may constitute the bulk of the diet for some

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prevalent mycophagous species (Maser et al. 1985; Taylor 1992), but in most cases they are only a seasonal resource (Lurz and South 1998; Pyare and Longland 2001; Hanson et al. 2003; Bertolino et al. 2004).

Edible fungi species are mainly saprotrophic and symbiotic. The first group grows on dead organic matter, making their cultivation easier than for other fungi. Actually, some 100 hundred species of saprotrophic fungi can be cultivated (Bao 2004). The three-quarters of the global production of cultivated fungi correspond to *Agaricus bisporus, Pleurotus* spp. and *Lentinula edodes* (Chang 1999). In the last 10 years, the production has seen a huge increase, mostly due to China, and each year the global business of the saprotrophic edible fungi is valued at around US$23 billion (Bao 2004). The largest edible fungus is a saprotrophic one, *Langermannia (Calvatia) gigantea* (giant puffball), measuring 2.64 m in circumference and weighting 22 kg (http://www.guinnessworldrecords.com/content_pages/ record.asp?recordid=47431). It has been estimated that a single basketball-sized *Langermannia gigantea* could produce enough spores in just two generations to theoretically produce a total volume of puffballs seven times the size of the earth.

Among the 2,500 species of edible fungi recorded, more than 400 are mycorrhizal species (EMF: edible mycorrhizal fungi) (Wang et al. 2002) and correspond to the majority of wild edible fungi, since saprotrophic fungi are mainly valued in their cultivated forms.

Most of the EMF are ectomycorrhizal species. However, there are some species, which are able to form ectendomycorrhizas and endomycorrhizas. This is the case for "desert truffles", *Terfezia claveryi* and *Picoa lefebvrei*, which form endomycorrhizas in natural conditions with *Helianthemum almeriense* (Gutierrez et al. 2003). Recently, it has been shown that some *Tuber* species are also capable of forming endomycorrhizas producing typical coiled hyphae when associated with orchids (see below; Selosse et al. 2004). However, the most valuable species are ectomycorrhizal, such as *Tuber magnatum, Tuber melanosporum, Tricholoma matsutake, Boletus edulis* s.l. and *Cantharellus cibarius* (Fig. 1). In contrast to the saprotrophic edible fungi, the culture of the symbiotic ones is more difficult. Only six species of symbiotic edible fungi can actually be successfully produced in presence of a plant: *Tuber melanosporum, T. aestivum, T. borchii, Terfezia claveryi, Lactarius deliciosus* and *Lyophyllum shimeji. Tuber maculatum* has been produced in one experiment in pots (Fig. 2; Fassi and Fontana 1969) and *Cantharellus cibarus* has also been produced in pots in a greenhouse (Danell and Camacho 1997). However, plantations with these species were not successful. Finally, for some of the most precious ectomycorrhizal edible fungi, such as *Tuber magnatum, Tricholoma matsutake* and *Boletus edulis* s.l., culture is as yet unsuccessful.

The production of EMF has been decreasing for the last 100 years. For example, *T. melanosporum* production fell from around 2,000 tons in the 1900s to sometimes less than 100 tons annually (Olivier 2000; Hall and Wang 2002). Similarly, current matsutake (*Tricholoma matsutake)* production in Japan is just 5% of the 1940s harvest (Ogawa 1978; Wang et al. 1997). The production and harvest of several *Cantharellus* species have seriously declined in some parts of Europe during the past several decades (Arnolds 1988, 1991, 1995, 2001; Gulden et al. 1992). For

Fig. 1 Fruit-bodies of some important edible mycorrhizal fungi. **a** *Tuber magnatum* ascocarps harvested in an italian truffle-ground (see: Mello et al, 2005) (Photo, V. Gavazza); **b,c** *Cantharellus cibarius* fructification harvested in Ardèche (France) (Photo, C. Murat); **d** *Boletus edulis* fructification harvested in Piedmont (Italy) (Photo, A. Vizzini)

instance, the number of locations where chanterelles fruit in the Netherlands has decreased by 60% in 20 years (Arnolds 1991, 1995; Arnolds and Jansen 1992; Jansen and Van Dobben 1987). How can we explain the decline of EMF production? There are several natural reasons that can explain the decline, such as the loss

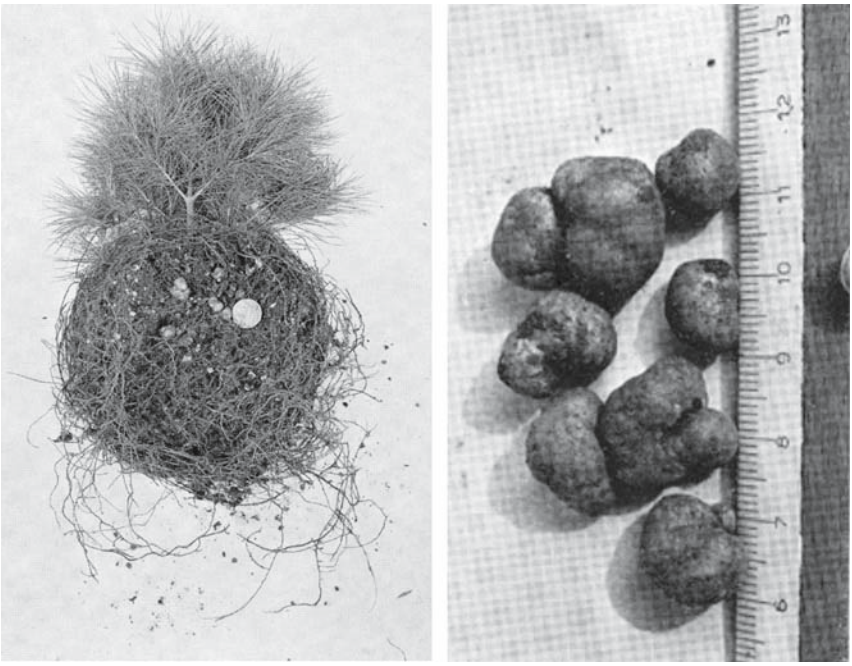

Fig. 2 *Tuber maculatum* ascocarps produced in a pot with *Pinus strobus* (Fassi and Fontana 1969)

of host plants within forests and global warming since the last ice age, as well as nonnatural (anthropic) reasons, such as deforestation, changed forest management practices, air pollution, soil acidification and fertilization, and soil compaction by hordes of pickers (Arnolds 1991, 2001; Peter et al. 2001; Hall et al. 2003).

Several researchers have found that picking has no negative impact on subsequent fruiting (Jahn and Jahn 1986; Arnolds 1991; Egli et al. 2006). In particular, two studies (mainly focused on *Cantharellus* species) conducted by the Oregon Mycological Society (Norvell 1995; Norvell and Roger 1998; Norvell et al. 1996) and by Swiss mycologists (Egli et al. 2006) have highlighted that long-term and systematic harvesting reduces neither the future yields of fruit bodies nor the species richness of wild forest fungi, irrespective of whether the harvesting technique was plucking or cutting. Forest floor trampling can reduce fruit body numbers, but data by Swiss researchers show no evidence that trampling damaged the soil mycelia in the studied time period (Egli et al. 2006). Search methods, however, can have some impact. Studies by Pilz and Molina (2002) of raking forest litter to find young *Tricholoma matsutake* fruit bodies suggest that raking into the mycelial layer can interfere with fruiting for several years, but that recovery is hastened by replacing the duff.

Fungi like truffles and boletes are eaten for their organoleptic qualities and for their nutritional value. They are low in fat and contain essential amino acids and useful mineral. Moreover, it is known that around 6% of edible mushrooms also have medicinal properties; some of them are used traditionally in Chinese medicine (Bao 2004). Of the about 200 medicinal fungi reported in literature only 5% are

ectomycorrhizal (Reshetnikov et al. 2001; Bao 2004). This is probably an underestimate since research efforts have concentrated on saprotrophic species that can be cultivated, thus providing a guaranteed supply and uniformity of product. *Volvariella volvacea, Lentinula edodes, Flammulina velutipes, Ganoderma lucidum, Hypsizygus marmoreus, Pleurotus* spp., *Tremella* spp. and *Auricularia* spp. are widely cultivated edible fungi with medicinal properties. The responsible bioactive compounds belong to several chemical groups; very often they are polysaccharides or triterpenes. One species can possess a high variety of bioactive compounds, and therefore of pharmacological effects. The best example is *Ganoderma lucidum*, which not only contains >120 different triterpenes, but also polysaccharides, proteins and other bioactive compounds (Kim and Kim 1999; Zhou and Gao 2002). However, the contribution to human health is difficult to estimate and has received little attention. The number of mushrooms with known pharmacological qualities is low and macromycetes are still far from being thoroughly studied (Rai 1997; Wasser and Weis 1999; Reshetnikov et al. 2001; Molitoris 2002; Wasser 2002; Lindequist et al. 2005; Zaidman et al. 2005; De Roman et al. 2006).

In addition to the main groups of saprotrophic and symbiotic edible fungi, there are some phytoparasitic species, such as *Armillaria* spp., which produce edible fruiting bodies. On the other hand, some peoples eat plant material infected with pathogenic fungi. In Mexico, maize cobs infected with the smut fungus *Ustilago maydis* (Basidiomycota, Ustilaginales) are consumed in large quantities, both fresh and canned, and are highly appreciated as a food delicacy. Young galls induced by the basidiomycete on immature corn ears are called in Aztec language, the "nahuatl", "uitlacoche" or "cuitlacoche". Huitlacoche remains a regional speciality because it is better fresh, but it has also been canned or frozen for export (Villanueva 1997; Pataky and Chandler 2003). The "lobster mushroom" (*Hypomyces lactifluorum*, Ascomycota, Hypocreales), which is a mycoparasite of macrofungi, particularly Russulaceae (*Lactarius* and *Russula*), is another example. This species is eaten in North America (Rogerson and Samuels 1994; Rochon, personal communication; http://botit.botany.wisc.edu/toms_fungi/aug2001.html).

The aim of this chapter is to provide an overview of the current knowledge on the EMF. The advances recently reached, thanks to the fungal genome projects of *Laccaria bicolor* (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/), *Tricholoma matsutake* (http://www.genomeweb.com/) and *Tuber melanosporum* (http://www.international.inra.fr/research/truffles_increasingly_rare), are providing new insights into the still largely unknown biology of these organisms.

2 From Morphological to Molecular Identification; *Tuber* and *Boletus* – A Two Case Study

Edible fungi must be first distinguished from the poisonous species. There are no reliable rules about which mushrooms are safe to eat and which are poisonous. Searching on the web for identification of edible fungi, 22 books dealing with the

macroscopic as well as the microscopic features of the mushrooms are listed at the website: www.fungi.com/books/fieldguides.html.

Theoretical debates on the species concept in fungi and how to recognize a fungal species are interesting points brilliantly discussed by Taylor et al. (2000). A phylogenetic approach based on concordance of multiple gene genealogies, morphology and reproductive behavior is the key to recognize fungal species. Nevertheless, for fruiting bodies, molecular identification based on the ITS region of ribosomal DNA, together with morphological identification has been often successful (Dahlberg et al. 1997; Mello et al. 1999).

For decades, mushroom identification has been based on macro- and micromorphological (microscopic examination of "tissues", spores and sporing structures), physiological and chemical characteristics of specimens. For the most part, these systems still work quite well. They provide inexpensively accurate species identification, are not very time-consuming, and require little equipment beyond a light microscope and few chemical reagents. A major drawback of the traditional identification methods is that they require some technical training in order to acquire the skills necessary to identify fungi: so the identification of fungi has always been considered difficult and sometime discouraging. In addition, morphological characters often overlap or can be difficult to record precisely and there is the need for standardization. In the last decade, new tools based on DNA analysis have led to major improvements in the identification, classifications and phylogeny of fungi. Problematic and ambiguous morphological features are evaluated by comparison of phylogenetic hypotheses based on morphology with those inferred from molecular characters. Molecular tools are commonly used to identify plant pathogenic fungi and have also been applied to truffle species in order to detect which species are used in prepared foods. The practical application of these tools for identifying and characterizing edible ectomycorrhizal macrofungi has still to be explored.

Truffles belong to the genus *Tuber* (Tuberaceae), one of the few ectomycorrhizal Ascomycetes even if other Ascomycetes are considered truffles, such as the desert truffles (Kirk et al. 2001). While in traditional classification systems they were included in the order Tuberales, together with all hypogeous Ascomycetes, today they are placed in the order Pezizales (O'Donnel et al. 1997; Percudani et al. 1999; Eriksson 2006; Mello et al. 2006a): this includes both hypogeous and epigeous fungi, with either saprotrophic, parasitic or symbiotic lifestyles, but which are all phylogenetically related (Hansen et al. 2001, 2005; Lutzoni et al. 2004; Ferdman et al. 2005; Vizzini 2005; Tedersoo et al. 2006). Hypogeous fruit bodies with concomitant loss of active ascospore discharge have been proved to have evolved independently several times from apothecial epigeous Pezizales.

Truffles live all over the world, distributed especially in many regions of the northern hemisphere. They have been found as far north as Gotland Island, in Sweden. Mycologists have described around 200 European species, varieties and forms of *Tuber* over the centuries (from the eighteenth to the twentieth). The various events linked to the species and their synonyms are summarized in a monograph of the European species of *Tuber* where the authors consider only 28 species to be valid (Ceruti et al. 2003).

Specialists generally recognize the morphological features of truffles and ascomata by a combination of peridial and sporal features. However, sometimes, identification is unreliable and this is a problem for edible *Tuber* species, especially for *T. magnatum* Pico, the "white truffle", and *T. melanosporum* Vittad., the "black truffle", which are in great demand by the food market in many countries because of their special taste and smell. *Tuber magnatum* has morphological features quite similar to the so-called *T. borchii* complex species, grouping "whitish" truffles (*T. borchii, T. puberulum, T. dryophilum* and *T. maculatum*) and characterized by less taste and a lower commercial value. Similarly *T. melanosporum* shares morphological features with *T. brumale* and the Asiatic *T. indicum*. Biochemical tools, such as one-dimensional gel electrophoresis with total protein (Mouches et al. 1981; Dupré et al. 1985) and isoenzyme analysis (Pacioni and Pomponi 1991; Gandeboeuf et al. 1994; Urbanelli et al. 1998) were the first to be used to verify the morphological identification of truffles. However, the protocols developed with these techniques are not quick and rely on high quality material. Since 1990, molecular tools have been used for identifying truffles. Before developing specific molecular probes, multi-loci analyses such as RAPD (random amplified polymorphism DNA) and RAMS (random amplified microsatellites sequences) were used (Lanfranco et al. 1993; Longato and Bonfante 1997). The fingerprints produced by these techniques were very complex to be compared, even if they identified *Tuber* species. When the attention of scientists moved from the inter- to intraspecific variability analysis, and the sequencing technology became more feasible, ITS was sequenced from many isolates of the same species. The alignment of these sequences revealed that ITS is variable enough to differentiate *Tuber* species. A pair of primers was designed to discriminate, in a single PCR experiment, *T. magnatum* from the "whitish" truffles (Table 1; Amicucci et al. 1998; Mello et al. 2000). As well as for the white truffle, specific primers were successful in distinguishing *T. melanosporum* from *T. brumale* and the Asiatic *T. indicum*, both of which are used in food in place of *T. melanosporum* (Table 1; Rubini et al. 1998; Paolocci et al. 2000). Detection of contaminant species in processed foods, sold as containing *T. melanosporum*, is now possible by PCR-RFLP, using a SNP on the mitochondrial Lsu-rDNA (Mabru et al. 2004).

Because of the high commercial profits involved in the business of *T. magnatum* and *T. melanosporum* and the high demand of these truffles, research programmes for large-scale mycorrhizal production have been elaborated in southern European countries to increase truffle production (Chevalier 1994). Since the 1970s, inoculated mycorrhizal seedlings produce *T. melanosporum* after 5–10 years (Le Tacon et al. 1988). At present, more than 80% of the French production of this truffle comes from artificial truffle-grounds. In addition to *T. melanosporum, T. borchii* and *T. uncinatum* are also collected from artificial truffle-grounds; by contrast, the production of *T. magnatum* comes only from natural truffle-grounds, although some sporadic records of samples collected from artificial truffle-grounds have been reported.

The identification of *Tuber* species during their symbiotic phase by the same methods developed for fruiting bodies (Rubini et al. 2001; Mello et al. 2001) has

Table 1 Non exhaustive list of specific primers to identify some *Tuber* spp

Species	Locus	Primers name	Reference
T. uncinatum–T. aestivum	ITS	TuITS1-TuITS4	Luis (2000)
	ITS	UncI-UncII	Mello et al. (2002)
	SCAR	Tu1400f-Tu1400r	Luis (2000)
	SCAR	Tu1800f-Tu1800r	Luis (2000)
T. melanosporum	ITS	ITSML-ITS4LNB	Paolocci et al. (1999)
	ITS	MELF-MELR	Douet et al. (2004)
T. brumale	ITS	ITSB-ITS4LNB	Paolocci et al. (1999)
	ITS	SYLV1-SYLV2	Douet et al. (2004)
T. indicum	ITS	ITSCHCH-ITS4LNB	Paolocci et al. (1999)
	ITS	IndF1-IndR	Mabru et al. (2001)
	ITS	IndF2-IndR	Mabru et al. (2001)
T. magnatum	ITS	ITSMAGN-ITSBACK3	Rubini et al. (2001)
	ITS	TmagI-TmagII	Amicucci et al. (1998)
	ITS	P7-M3	Mello et al. (2001)
	SCAR	TAR I-TAR II	Amicucci et al. (1996)
T. puberulum	ITS	TpuI-TpuII	Amicucci et al. (1998)
T. dryophilum	ITS	TdryI-TdryII	Amicucci et al. (1998)
T. maculatum	ITS	TmacI-TmacII	Amicucci et al. (1998)
T. borchii	ITS	TboI-TboII	Amicucci et al. (1998)
	ITS	TBA-TBB	Mello et al. (2000)

led to the conclusion that, at least for *T. magnatum*, only 20% of the analyzed plants was successfully colonized, suggesting that *T. magnatum* has a low mycorrhizal efficiency (Mello et al. 2001). After a long-term survey of about 1,200–1,600 tips thought to be colonized by *T. magnatum*, molecular analyses clearly showed that samples kept in controlled situations, i.e., a growth chamber or greenhouses, lead to a positive amplification with ITS specific primers. Failure to obtain *T. magnatum* mycorrhizas in field conditions may thus be due to the fact that this species has to compete with more aggressive truffles, such as *T. maculatum* and *T. borchii*. These competitive species may already occur in nurseries or be erroneously mixed in the inoculum during large-scale experiments. The research of *T. magnatum* fruiting bodies as well as of mycorrhizae (through morphological and molecular approaches) in a natural truffle-ground has revealed that this fungus invests more in fruiting body formation than in root colonisation (Murat et al. 2005). In fact, *T. magnatum* mycorrhizae was found to be very rare in the field, challenging the importance of the truffle's symbiotic phase.

Beside the precious species identification, another challenge has been the controversial taxonomic position of two taxa that have a moderate commercial value, *T. aestivum* Vittad. and *T. uncinatum* Chatin. Although they are very similar, some ecological features, geographical distributions and smell and taste are distinctive. After two publications leading to controversial results (Mello et al. 2002; Paolocci et al. 2004), but providing the first ITS sequences of these taxa, Weden et al. (2005) showed that the height of the spore reticulum, considered the most useful

morphological characteristic for distinguishing the two taxa, is not diagnostic, and that it is not possible to separate *T. aestivum* from *T. uncinatum* on the basis of this character.

The increase of nucleotide sequences of truffles together with the building of phylogenetic trees has helped to resolve the problem of their interspecific differences, limiting frauds, and answering questions related to their taxonomy. However, some identification problems still exist. In fact, new sequences from *T. borchii* submitted in the NCBI database by Halász et al. (2005) do not correspond to the sequences attributed in the past to *T. borchii* (Mello and Zambonelli, personal communication). This new problem requires exchanges of specimens among the groups involved in the truffle taxonomy for an unambiguous identification.

2.1 As Tuber, the Genus Boletus Raises Taxonomic Problems and Offers an Interesting Case Study

The genus *Boletus* belongs to the family Boletaceae, order Boletales, and is a complex of many sections. Among them, the section *Boletus* Singer (=*Edules* Fr. 1838) has long been considered a complex of hardly distinguishable species. In all, 21 taxa, including species, varieties and forms, have been ascribed to this complex (Lannoy and Estades 2001). All the species included are characterized by white-textured unchanging flesh, never tending towards the blue, more or less reticulated stipe, and initially white pores, which later become yellowish and then greenish. Among them, *B. edulis* Bull.: Fr. sensu stricto, *B. aereus* Bull.: Fr, *B. pinophilus* Pilát et Dermek and *B. aestivalis* (Paulet) Fr. (all classified as *B. edulis* sensu lato) are considered valid taxa by the mycological community and represent the most frequently eaten fungi, among those harvested in natural conditions in Europe. Mushrooms belonging to the *B. edulis* s.l. are used in huge quantities for the production of dried mushroom-soups. Unfortunately, the addition of the cheaper *Suillus luteus* ("slippery jack") to the more expensive *B. edulis* s.l., the king bolete, macroscopically not closely related, is a frequent practice, because microscopic examination is not applicable in such highly processed products as dried soup, pâtés or pies.

DNA-based methods have now been applied to the detection of king bolete and slippery jack in food samples (Moor et al. 2002). The internal transcribed spacer (ITS) of the ribosomal DNA was sequenced from *B. edulis* s.l. and *Suillus* species. From the sequence alignment, the reverse primers were set specifically in the variable ITS1 region for *Boletus* or *Suillus* and used in Multiplex PCR, in conjunction with the ITS1-F basidiomycete primer (Gardes and Bruns 1993).

This multiplex assay allows the detection of fraudulent additions of no more than 1–2% of *Suillus luteus*, and therefore sensitive enough for quality control. In addition to this problem, *B. edulis* s.l. fruit bodies have contrasting fungal features. These fruiting bodies are, in fact, in high demand as mushrooms with a pleasant flavor and texture, but at the same time, they have been shown to induce allergic IgE-mediated

symptoms either through inhalation, ingestion or contact (Helbling et al. 2002). When investigating whether *B. edulis* induces distinct allergens, these authors showed that the fungus causes food allergy in two ways: first, causing systemic symptoms probably due to cross-reactivity to inhaled, atmospheric allergens, and second, due to a digestion-stable 75 kDa protein (Helbling et al. 2002). In order to guarantee safe naturally derived food, Mello et al. (2006b) developed specific primers for the unambiguous detection of *B. edulis* sensu stricto, *B. aereus, B. pinophilus* and *B. aestivalis*. In this investigation, ITS analysis gave rise to a phylogenetic tree where four clusters were distinguishable in the *B. edulis* s.l., according to the morphological criteria used in this work and in Leonardi et al. (2005). Furthermore, it allowed the definition of the relationships of *B. edulis* with *B. violaceofuscus*, a spectacular violaceous-tinted bolete. Fragments of *B. violaceofuscus*, Chiu (1948), originally described from China and also living in Japan (e.g., Hongo 1960; Imazeki and Hongo 1989) and considered as belonging to the section *Boletus* (Singer 1986), are currently imported into Europe from China and frequently sold among dried mushrooms of *B. edulis* s.l. (Floriani et al. 2000). Interestingly, Mello et al. (2006b) found that *B. violaceofuscus* was wrongly attributed to the section *Boletus*, opening the question whether it belongs to the genus *Boletus*, too. In an attempt to study the close relationship between *B. violaceofuscus* and *B. separans*, an Eastern North America bolete, by pigment chemistry and 28S phylogenetic analyses, Simonini et al. (2001) suggested that these species are outside the section *Boletus* and the genus *Boletus*. Because of this finding, the sale of *B. violaceofuscus* as dried fruit bodies of the section *Boletus* has to be debated. To detect the fungus, a pair of primers specific for *B. violaceofuscus* has been designed by Mello et al. (2006b). The detection of this spectacular fungus, together with the probes aimed at discriminating the four species of the *Boletus edulis* s.l., opens the possibility of safely monitoring the market of these high priced fungi. As the paper by Helbling et al. (2002) does not give any taxonomic indication about *B. edulis* (sensu stricto or sensu lato) and since the individual allergenic responses could be the common effects of *B. edulis* s.s., *B. aereus, B. aestivalis, B. pinophilus*, further immunological experiments should be performed once it is possible to discriminate the four species of the *Boletus* s.l.

Since a large-scale reforestation with suitable plants and the harvest of fruit bodies are an important goal in forest management and cultivation (Hall et al. 1998), many attempts to cultivate edible boletes have been made. However, a protocol for their commercial production has not yet evolved and the results are very poor until now. Attempts to infect host plants in order to cultivate *B. edulis* s.l. gave several problems with different techniques (sporal inocula, mother plant technique); fruiting bodies being produced from transplanted plants infected with *B. edulis* s.l. have never been reported (Hall et al. 1998; Wang and Hall, 2004). In vitro mycelia have been produced in order to inoculate seedlings in controlled conditions and to increase production (Montemartini Corte and Vianello 1996). In some mountainous and hilly areas of Europe, this group of fungi is not only a natural product but also a source of seasonal income for the population. Salerni and Perini (2004) have investigated the effect of tree canopy and the presence/absence of litter on fruit body production of *B. edulis* s.l. in order to provide indications for the forest

management aimed at conserving the ideal habitat of this natural product. Once these technical problems are overcome, the availability of molecular tools will allow the certification of pure cultures of *B. edulis* as well as use-ready inocula to be used in long-term reforestation programs (Iotti et al. 2005).

3 The Life Cycle of Edible Mycorrhizal Fungi

3.1 General Considerations

Edible mushrooms have different life strategies mirroring their nutritional status, ranging from saprotrophic to parasitic and mycorrhizal. Irrespective of that, edible fungi produce conspicuous fruiting bodies where hyphae aggregate, produce pseudo tissues with differentiated compartments, develop specialized structures, and eventually differentiate meiotic spores. The life cycle of edible saprotrophic fungi is well known since the different phases of their life cycle can be reproduced in vitro including fruiting bodies formation. On the contrary, the life cycle of EMF is more difficult to investigate experimentally. Indeed, to accomplish their life cycle EMF need to establish a symbiotic relationship with a narrow range of host plants. Three phases are usually identified in their life cycle: (1) a vegetative stage which corresponds to the hyphal growth in the underground soil ecosystem; (2) a symbiotic stage when the mycorrhizal association is established; and (3) a reproductive stage leading to the organization of hypogeous or epigeous fruiting bodies. The first two stages can at least in part be mimicked in laboratory conditions.

The accomplishment of the life cycle under laboratory conditions is a crucial goal to unravel the biology of EMF. To our knowledge the in vitro life cycle of edible mycorrhizal fungi with formation of fruiting bodies in a Petri dish has been realized only for one species, *Laccaria laccata* (Unestam and Stenstrom 1989). In our laboratory, A. Fontana obtained, in 1969, the first and unique production of a truffle species, *Tuber maculatum*, in controlled conditions using pots with *Pinus strobus* (Fig. 2; Fassi and Fontana 1969). Unfortunately, this experience has never been repeated. In 1997, Danell and Camacho published the first successful fruitbody formation of *Cantharellus cibarius* in a greenhouse, hosted by pine seedlings only 16 months old. However, it is not actually known whether the cultivation of *C. cibarius* is feasible.

3.2 Edible Mycorrhizal Fungi: Basidiomycetes

Most of the edible mycorrhizal species are Homobasidiomycetes, such as porcini (*Boletus edulis* s.l.), chanterelles (*Cantharellus cibarius*), and matsutake (*Tricholoma matsutake*). Basidiomycete fungi are largely heterothallic, and a remarkable feature

of this group is that they have evolved multiallelic mating type genes; as a result, some of them have many thousands of different mating types (Casselton and Olesnicky 1998). Genes at two unlinked loci, known as A and B, determine the mating type in the mushroom. These genes encode both peptide pheromones and transmembrane receptors (Casselton and Olesnicky 1998). Among the ectomycorrhizal fungi, the homobasidiomycete *Hebeloma cylindrosporum* forms inedible fructification. However, it is currently considered a model species: its entire life cycle, including fruit body formation, can be obtained under axenic conditions in the laboratory using defined culture media (Debaud and Gay 1987; for a review, see: Marmeisse et al. 2004). *H. cylindrosporum* has a typical bifactorial heterothallic life cycle with A and B mating-type controlling the mating between haploid mycelia (Debaud et al. 1986). Germination of basidiospores produces haploid homokaryotic mycelia that have symbiotic capacities (Debaud et al. 1988). Two compatible homokaryons establish a dikaryon mycelium, which can be used to inoculate to sterile *Pinus pinaster* seedlings growing on a vermiculite substrate saturated with a defined medium. Ectomycorrhizas are formed within 3–6 months followed by sporulating fruit bodies (Debaud and Gay 1987). Two haploid nuclei fuse inside basidia and the resulting diploid nucleus enters in meiosis to produce four haploid nuclei; they undergo a successive mitosis leading to the formation of basidiospores, each containing two identical nuclei. EMF might have a similar life cycle with an A and B mating type. However, for the moment, experimental tools to check such hypothesis in species such as *Boletus* spp. and *Tricholoma matsutake* are still lacking.

On the other hand, recent analysis made in vivo allowed the unraveling of some steps of the life cycle of *T. matsutake*. This species forms a shiro, that is a massive mycorrhizal-mycelial aggregate such a solid and tight aggregate (Hamada 1970). The border between the shiro and its surrounding area can be easily recognized. Murata et al. (2005) found that *T. matsutake* fruit bodies produced in the same shiro and the spores in the same fruit bodies were genetically diverse, indicating the occurrence of genetic mosaics in shiro. These authors suggested that the dispersal of spores through sexual reproduction is important. These results could be important to protect this species, which exhibits a production decline, since *T. matsutake* is often harvested before the dispersal of spore and this could explain the production decrease.

3.3 Edible Mycorrhizal Fungi: Truffles as an Example of Ascomycete

Some species of EMF, such as truffles, belong to Ascomycota. During their life cycle, Ascomycetes must choose between sexual and asexual development, and they use nutritional, temperature and light clues to make this decision (Nelson 1996). Sexual recognition is controlled by the mating type locus (for a review, see: Coppin et al. 1997), whereas vegetative recognition is controlled by vegetative (or heterokaryon) incompatibility systems (for a review, see: Saupe 2000). Most filamentous Ascomycetes spend nearly their entire existence as haploids, and many form asexual spores (conidia) that serve as vegetative propagules (conidia) or as

male, fertilizing parents (spermatia) in sexual crosses (Alexopoulos and Mims 1979). The sexual development of filamentous Ascomycetes is characterized by the formation of a complex fruiting body. Three different basic sexual reproduction strategies are known, i.e. homothallism, pseudohomothallism and heterothallism (Nelson 1996; Pöggeler 2001). Individual haploid mycelia of homothallic and pseudohomothallic (haploid but heterokariotic) fungi are self-fertile (Pöggeler 2001). In the young ascus, two haploid nuclei fuse to form a diploid nucleus from which sexual progeny as ascospores take origin. The ascospores are forcibly discharged from the fruiting bodies formed by most filamentous Ascomycetes (Nelson 1996). When conditions are favorable, ascospores start the germination process. In the case of mycorrhizal fungi, the germinating mycelium has to reach root tips to form mycorrhizal symbiosis.

Recently, Rubini et al. (2005) and Paolocci et al. (2006) used polymorphic microsatellites to compare the allelic configuration of ascospores with those from the network of surrounding hyphae and mycorrhiza of *T. magnatum*. Truffles are expected to have a very closed mating system, such as homothallism or even exclusively selfing (Bertault et al. 1998; Mello et al. 2005). Indeed, since 1998 and the paper by Bertault et al. (1998), all studies performed on *T. melanosporum* and *T. magnatum* ascocarps never identified heterozygotes (Frizzi et al. 2001; Bertault et al. 2001; Murat et al. 2004; Mello et al. 2005). Rubini et al. (2005) and Paolocci et al. (2006) showed that recombination occurs in *T. magnatum*, and they explain the lack of heterozygosity by the fact that "DNAs extracted from the ascocarps are almost exclusively that of the maternal parent". Indeed, Paolocci et al. (2006) detected heterozygosity only from DNA extracted from ascospores. This hypothesis questions the validity of all the papers until now that have focused on *T. magnatum* truffle genetic diversity and population genetics.

On the basis of the existing data, Paolocci et al. (2006) suggest a new model for the *T. magnatum* life cycle (Fasolo-Bonfante and Brunel 1972; Lanfranco et al. 1995; Mello et al. 2005; Rubini et al. 2005; see Fig. 1 in Paolocci et al. 2006). First, asci are released following ripening of the ascocarp and, in proximity to the host plant root tips, ascospores germinate to produce primary homokaryotic (haploid) mycelium which form ectomycorrhizae. From them, extramatrical primary mycelia develop and spread into the soil. Recently, Suz et al. (2006) confirmed that *T. melanosporum* mycelium spread throughout the first 35 cm of the soil profile below ectomycorrhizae. For *T. magnatum*, details of the fertilization process are unknown, but by analogy to other Ascomycetes, a spermatization process seems likely (Paolocci et al. 2006). These authors suggested that the function of a male gamete might be filled up by any detached cell, e.g., a hyphal fragment, conidium, or ascospore. Urban et al. (2004) report the finding of conidia of *T. borchii* and *T. oligospermum*, although not coming from *T. magnatum*, these data could support the hypothesis of the spermatization in the cycle of *T. magnatum*. Eventually, the ascocarp primordia develop. According to this model, the ascogenous heterokaryotic hyphae resulting from the fertilization process are surrounded by homokaryotic vegetative hyphae of maternal origin.

Unfortunately, such hypotheses cannot be verified experimentally due to the lack of an experimental system based on spore germination and on their crossing to

the resulting mycelia. Actually, the mycelial in vitro growth is successful only for some *Tuber* species. In her pioneering research back to 1968, Anna Fontana described a protocol to obtain *Tuber* mycelia starting from fruit bodies. In the following years, the mycelial development of several *Tuber* spp. has been described, such as *T. borchii* (Fontana and Palenzona 1969) and *T. melanosporum* (Fontana 1971). The growth of the following *Tuber* spp. is currently available: *T. borchii, T. maculatum, T. melanosporum, T. aestivum, T. macrosporum, T. rufum* and *T. brumale* (Fontana and Palenzona 1969; Fontana 1971; Saltarelli et al. 1998; Iotti et al. 2002). Unfortunately, the mycelial growth of *T. magnatum* has not yet been obtained. The growth of truffle mycelium is generally slow; for example, Iotti et al. (2002) reported the mycelium of *T. rufum* as the fastest species (14 mm/week) and *T. melanosporum* as the slowest one (1.1 mm/week).

The first synthesis of truffle ectomycorrhiza in controlled conditions has been accomplished in pots. In 1962, Fassi and De Vecchi (1962) produced ectomycorrhizas of *T. maculatum* by using *Pinus strobus* as a host plant. This experimental system also led to the production of fruit bodies (Fig 2; Fassi and Fontana 1969). Other truffle mycorrhizas have been obtained with other species, such as *T. maculatum* on *Pinus strobus* and Euro-American poplar (Fontana and Palenzona 1969); *T. aestivum, T. brumale* and *T. melanosporum* on *Corylus avellana* (Palenzona 1969); *T. macrosporum* on *Corylus avellana, Quercus pubescens* and *Q. pedunculata* (Giovannetti and Fontana, 1980–81); *T. borchii* on *Tilia platyphyllos* (Sisti et al. 1998); *T. brumale* on *Tilia americana* and *Q. pubescens* (Giomaro et al. 2002). We recently discussed the importance of the symbiotic phase in the *T. magnatum* life cycle by analyzing the subterranean ectomycorrhizal community in a *T. magnatum* truffle-ground (Murat et al. 2005). In the screening of mycorrhizal tips, *T. magnatum* mycorrhizae were found to be very rare: it seems that the fungus invests more in fruit body formation than in root tip colonization (Murat et al. 2005). Furthermore, *T. magnatum* mycorrhizae were present in a nonproductive period (May) for *T. magnatum* as well as in a nonproductive area (see Mello et al. 2005), suggesting that there is not a direct linkage between mycorrhizae and fruit bodies, as was found for *Suillus grevillei* (Zhou et al. 2001). All these observations challenge the importance of a truffle symbiotic phase, suggesting that truffles may be more plastic in their metabolism than expected, i.e., they could survive in the soil as saprotrophic mycelia. On this issue, a recent report shows that some *Tuber* species can also be endophytic inside roots of chlorophyllous and achlorophyllous plants such as orchids, suggesting a possible role as a bridge between mycoheterotrophic species and ectomycorrhizal trees (Selosse et al. 2004).

3.4 The Morphogenesis

Fruiting body formation is probably the most complex stage in the life cycle of fungi. It involves dramatic changes of the growth pattern: from a loose mesh of undifferentiated hyphae a compact multihyphal structure is formed and devoted to

sexual reproduction. Saprotrophic macrofungi develop on a wide variety of substrates, from lignin- and cellulose-containing substrates to industrial and agricultural wastes, so the edible species are quite easily and successfully cultivated throughout the world. Mushroom cultivation presents an economically important biotechnological industry that has markedly expanded all over the world in the past few years with a production of about 5×10^6 tonnes fresh weight per year (Kües and Liu 2000). *Agaricus bisporus*, the button mushroom, is the most commercialized mushroom in the world and represents about 30% of the total world production of fungi (Olivier 1991).

By contrast, the situation is more complex for ectomycorrhizal fungi, because the fruiting body represents a unique step of a complex life cycle, which requires the symbiotic association with a plant host. The successful production of fruiting bodies in vitro has only been reported for four ectomycorrhizal species (see above): *Tuber maculatum, Hebeloma cylindrosporum, Laccaria laccata, Cantharellus cibarius* (Fassi and Fontana 1969; Debaud and Gay 1987; Unestam and Stenstrom 1989; Danell and Camacho 1997). Even other precious fruiting bodies of mycorrhizal fungi such as *Morchella* spp., *Tuber* spp., *Boletus edulis* s.l., *Cantharellus cibarius* and *Tricholoma matsutake* still have to be collected from the wild with variable and unpredictable harvesting from year to year (Redhead et al. 1997; Yamanaka 1997). The economic value of these fungi is triggering the efforts of the scientific community to increase the very few data available so far on the molecular events leading to the fruiting body formation of truffle species and other ectomycorrhizal fungi.

Most efforts have been devoted to search for genes controlling the developmental switch between the vegetative and the reproductive stage and determining the structure and the final shape of fruiting bodies. Current knowledge shows that fruiting genes are controlled by mating-type genes and by transcriptional regulators in *Schizophyllum commune* (Wessels et al. 1985). In other saprotrophic Basidiomycetes, such as *Coprinus cinereus* (Boulianne et al. 2000), *Lentinula edodes* (Leung et al. 2000), *Flammulina velutipes, Agaricus bisporus* (De Groot et al. 1997), *Volvariella volvacea* (Chen et al. 2004) and *Agrocybe aegerita* (= *A. cylindracea*) (Sirand-Pugnet et al. 2003), fruiting body-specific or fruiting body-induced genes have been identified by different molecular techniques. In many cases, these genes belong to the family of hydrophobins, small cystein-rich proteins that coat the aerial structures with a hydrophobic water-repellent layer. In addition, a number of genes acting in substrate degradation are available, such as laccases. A function in the formation of extracellular pigments, coupled to oxidative polymerization of cell wall components to strengthen cell-to-cell adhesion, has also been suggested for *L. edodes* Lac1 and Lac2 laccases (Zhao and Kwan 1999). Hyphal aggregation, a crucial step in fruiting body formation, has been proposed to be mediated by a protein similar to haemolysin in *A. aegerita* (Fernandez Espinar and Labarere 1997), by lectins and galectins identified in *A. bisporus* and *C. cinereus* (Crenshaw et al. 1995; Cooper et al. 1997), and by a putative Eln3 enzyme located in the plasma membrane for *C. cinereus* (Arima et al. 2004).

Another class of enzymes of primary importance in fruiting body morphogenesis are chitin syntases (Chs). Chs genes are considered as molecular markers of

development both in filamentous and in yeast growth patterns. Each member of this multigenic family is supposed to have a distinctive functional role during the fungal developmental process. As regards edible fungi, Class III and IV Chs mRNAs are differentially expressed in a maturation stage-dependent manner in *T. borchii* fruiting bodies and accumulate in sporogenic and vegetative hyphae, respectively (Balestrini et al. 2000). Even in *Agaricus bisporus*, an expressed sequence tag similar to a class V *Chs* gene was found to be more expressed in developing basidioma (Ospina-Giraldo et al. 2000).

Environmental conditions including light, temperature, humidity and nutrients availability play a decisive role in determining whether a fruiting body will be formed. Fruiting is typically induced by reducing temperature, high humidity, neutral or slightly acidic pH and pulses of low energy light, but a universal set of conditions that lead to fructification in all fungi cannot be defined. For example, light is implicated in the fruiting of several *Coprinus* species, *Pleurotus ostreatus* and *F. velutipes*, but does not affect the fruiting of *A. bisporus* (Wessels 1993). Even a hypogeous organism, like *T. borchii*, responds to blue light by inhibiting apical growth (Ambra et al. 2004), but there is still no evidence that light can play a role in truffle fructification.

Among the main environmental factors which trigger the fungal fruiting in both Asco- and Basidiomycetes, a crucial role is played by the physiological condition and nutritional state of the mycelium (Flegg and Wood 1985). High ammonium concentrations are known to act as inhibitors of meiosis and sporophore morphogenesis (Ewaze et al. 1978). An investigation of the expression profile of glutamine synthetase (GS) in the whitish truffle *T. borchii* (Montanini et al. 2003) showed that GS, which is the primary NH_4^+ assimilating enzyme in mycelia grown under N-limiting conditions, is highly expressed in fruiting bodies. This could mean both that fruiting bodies experience N deprivation in a developmental stage that requests a high biosynthetic activity and those fruiting bodies have to keep free NH_4^+ at very low concentration.

Relocation of nutrients is thought to occur throughout the mycelium to favor the primordia formation. In *C. cinereus*, during the transition from vegetative growth to fruiting body development, a breakdown of storage polysaccharides is observed (Kües and Liu 2000). It provides the metabolic energy for mushroom development. A similar relocation of nutrients seems to occur in *T. borchii* as well. Recently, an EST-cDNA arrays approach provided the first global view of events controlling fruiting in *T. borchii* (Lacourt et al 2002; Gabella et al. 2005). In this fungus, glycolysis is downregulated during fruit body maturation (Saltarelli et al. 1998), but it is the catabolism of lipids accumulated in vegetative mycelium which is supposed to sustain the constant carbon flux needed for fruit body construction and maturation. Two-carbon compounds resulting from this fatty acid degradation are probably assimilated into the tricarboxylic acid cycle through the glyoxylate cycle steps catalyzed by isocitrate lyase and malate synthase. Moreover, products related to lipid degradation (i.e., alcohol 1-octen-3-ol) are present in the volatile organic compounds (VOCs) responsible for the “fungal smell” in *T. borchii* (Richard Splivallo, personal communication).

Fruiting is typically induced, after vegetative growth, by reducing the temperature, e.g., in *A. bisporus* to 16–18°C, in *C. cinereus* to 25–28°C and in *F. velutipes* to 18°C (Flegg and Wood 1985; Williams et al 1985; Scrase and Elliott 1998; Kües 2000). The upregulation of the dehydrin-like TbDHN1 in *T. borchii* fruiting body (Abbà et al. 2006) is probably strictly linked to the exposure of fruiting bodies to low temperatures during the autumn/winter season.

Fruiting body production is not well understood to date. A lot of genes and proteins with an unknown biological function have been discovered to be highly expressed during fruiting body formation and maturation in *T. borchii, L. edodes* and *F. velutipes* (Zeppa et al. 2002; Lacourt et al. 2002; Pierleoni et al. 2004; Palma et al. 2005; Gabella et al. 2005; Miyazaki et al. 2005; Yamada et al. 2005). A genetical approach, such as mutants and gene deletion, could help to unravel their role in the developmental process underlying fruiting.

T. borchii was genetically transformed both by *Agrobacterium*-mediated transformation (Grimaldi et al. 2005) and through liposome-mediated delivery of genetic material with mycelial protoplasts isolation and fusion with liposome (Poma et al. 2005, 2006), but the deletion of a specific gene in truffle has never been attempted.

3.5 The Spore Dispersal

The fruit bodies of EMF have particular organoleptic qualities that are not only appreciated by humans but by animals, too. Probably these organoleptic qualities are the consequence of evolution to use animals for the spore dispersal. The epigeous species can disperse their spores both by wind and mammals dispersal. It is known that spores of edible mycorrhizal fungi can be dispersed by wind and diffuse over long distances (Allen et al. 1992). For example, Ashkannejhad and Horton (2006) suggest that suilloid fungi are uniquely adapted for long-distance dispersal. In contrast, the true (*Tuber* spp.) or false (e.g., *Rhizopogon* spp., *Terfeziaceae* spp.) truffles have hypogeous sporocarps and have lost the ability to propel their spores into the air. The long-distance dispersal of these species is thought to be via mammals (Luoma et al. 2003). Indeed, hypogeous sporocarps produce odors that attract deers, elks, mountain goats, wild bears, rodents and marsupials that dig up the sporocarps, consume them and disperse their spores when they defecate (Thiers 1984; Johnson 1996; Luoma et al. 2003; Bertolino et al. 2004; Caldwell et al. 2005; Ashkannejhad and Horton 2006). Recently, Caldwell et al. (2005), during inoculation tests of *Picea rubens* seedlings, have demonstrated that passage through the digestive tract of North American flying squirrels (*Glaucomys sabrinus*) may enhance germination and inoculation potential of fruit bodies spores belonging to hypogeous ascomycetes (*Elaphomyces*), although actively growing mycelium in soil may be the primary and most effective means by which trees develop ectomycorrhizae under natural conditions.

4 Conclusion

Even if in the last decade our knowledge on the biology, genetic and morphogenesis of EMF has increased, several aspects of their biological cycle are still unclear, due to the lack of a model species. Recently, the genome sequence of *Populus trichocarpa*, a host of EMF, has been sequenced (Tuskan et al. 2006). As a consequence of the genome sequence of the poplar, the United States Department of Energy Joint Genome Institute (JGI) sequenced several known *Populus* associates, the endomycorrhizal glomeromycete fungus *Glomus intraradices* and the ectomycorrhizal basidiomycete *Laccaria bicolor* (Martin et al. 2004). This ectomycorrhizal species does not produce appreciated edible fruit bodies, but information obtained from its genome should be transposed to EMF phylogenetically related species such as *Laccaria laccata*. Currently, only the genome of *Tricholoma matsutake* has been sequenced. Takara Bio Inc., a company based in Shiga Prefecture's Otsu City (Japan), announced in December 2004 that it had succeeded for the first time in the world in deciphering the genome sequence of matsutake mushroom. The company's research institute began trying to decipher the fungal genome in May 2004, and in half a year it has succeeded in obtaining data involving about 80% of the genome sequence (http://www.japancorp.net/Article.Asp?Art_ID=9009). Unfortunately, the genome sequence is not available to scientific community.

Recently, a consortium, headed by Francis Martin and involving French INRA, Italian and Belgium universities, launched a project aimed to sequence and study the truffle genome. A strain of *Tuber melanosporum* from the INRA collection in Clermont-Ferrand has been chosen for sequencing, which is expected to start in 2006 at the Génoscope–National Sequencing Centre, in Evry (France) (http://www.international.inra.fr/research/truffles_increasingly_rare). The truffle genome deciphering associated with new strategies for genetic transformation, breeding and in vitro culture will provide insights and potential solutions to protect and maximize the value of EMF.

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References

Abbà S, Ghignone S, Bonfante P (2006) A dehydration-inducible gene in the truffle *Tuber borchii* identifies a novel group of dehydrins. BMC Genomics 7:39

Alexopoulos CJ, Mims CW (1979) Introductory mycology, 3rd edn. Wiley, New York

Allen MF, Crisafulli C, Friese CF, Jeakins SL (1992) Re-formation of mycorrhizal symbioses on mount St Helens, 1980–1990- Interactions of rodents and mycorrhizal fungi. Mycol Res 96:447–453

Ambra R, Grimaldi B, Zamboni S, Filetici P, Macino G, Ballario P (2004) Photomorphogenesis in the hypogeous fungus *Tuber borchii*: isolation and characterization of Tbwc-1, the homologue of the blue-light photoreceptor of *Neurospora crassa*. Fungal Genet Biol 41:688–697

Amicucci A, Rossi I, Potenza L, Zambonelli A, Agostini D, Palma F, Stocchi V (1996) Identification of ectomycorrhizae from *Tuber* species by RFLP analysis of the ITS region. Biotech Lett 18:821–826

Amicucci A, Zambonelli A, Giomaro G, Potenza L, Stocchi V (1998) Identification of ectomycorrhizal fungi of the genus *Tuber* by species-specific ITS primers. Mol Ecol 7:273–277

Arima T, Yamamoto M, Hirata A, Kawano S, Kamada T (2004) The eln3 gene involved in fruiting body morphogenesis of *Coprinus cinereus* encodes a putative membrane protein with a general glycosyltransferase domain. Fungal Genet Biol 41:805–812

Arnolds E (1988) The changing macromycete flora in the Netherlands. Trans Br Mycol Soc 90:391–406

Arnolds E (1991). Decline of ectomycorrhizal fungi in Europe. Agric Ecosyst Environ 35:209–244

Arnolds E (1995) Conservation and management of natural populations of edible fungi. Can J Bot 73:987–998

Arnolds E (2001) Hoop vor de Hanekam. Coolia 44:48–56

Arnolds E, Jansen E (1992) New evidence for changes in the macromycete flora in the Netherlands. Nova Hedwigia 55:325–351

Ashkannejhad S, Horton TR (2006) Ectomycorrhizal ecology under primary succession on coastal sand dunes: interactions involving Pinus contorta, suilloid fungi and deer. New Phytol 169:345–354

Balestrini R, Mainieri D, Soragni E, Garnero L, Rollino S, Viotti A, Ottonello S, Bonfante P (2000) Differential expression of chitin synthase III and IV mRNAs in ascomata of *Tuber borchii* Vittad. Fungal Genet Biol 31:219–32.

Bao E (2004) Wild edible fungi: a global overview of their use and importance to people. FAO, Rome.

Bertault G, Raymond M, Berthomieu A, Callot G, Fernandez D (1998) Trifling variation in truffles. Nature 394:734

Bertault G, Rousset F, Fernandez D, Berthomieu A, Hochberg ME, Callot G, Raymond M (2001) Population genetics and dynamics of the black truffle in a man-made truffle field. Heredity 86:451–458

Bertolino S, Vizzini A, Wauters LA, Tosi G (2004) Consumption of hypogeous and epigeous fungi by the red squirrel (*Sciurus vulgaris*) in subalpine conifer forests. For Ecol Manage 202:227–233

Bertolino S, Vizzini A, Wauters LA, Tosi G (2004) Consumption of hypogeous and epigeous fungi by the red squirrel (*Sciurus vulgaris*) in subalpine conifer forests. For Ecol Manage 202:227–233

Boulianne RP, Liu Y, Aebi M, Lu BC, Kues U (2000) Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway. Microbiology 146:1841–53

Caldwell IR, Vernes K, Barlocher F (2005) The northern flying squirrel (*Glaucomys sabrinus*) as a vector for inoculation of red spruce (*Picea rubens*) seedlings with ectomycorrhizal fungi. Sydowia 57:166–178

Callot G (1999) La truffe, la terre, la vie. INRA, Paris

Casselton LA, Olesnicky NS (1998) Molecular genetics of mating recognition in basidiomycete fungi. Microbiol Mol Biol Rev 62:55–70

Ceruti A, Fontana A, Nosenzo C (2003) Le specie Europee del Genere *Tuber*. Museo Regionale, Torino

Chang ST (1999) World production of cultivated edible and medicinal mushrooms in 1997 with emphasis on *Lentinus edodes* in China. Int J Med Mushrooms 1:291–300

Chen S, Ge W, Buswell JA (2004) Biochemical and molecular characterization of a laccase from the edible straw mushroom, *Volvariella volvacea*. Eur J Biochem 271:318–328

Chevalier G (1994) Evolution des recherches sur les plants mycorhizes par la truffe et perspectives de development. Giorn Bot Ital 128:7–18

Chiu WF (1948) The boletes of Yunnan. Mycologia 40:199–231

Cooper DN, Boulianne RP, Charlton S, Farrell EM, Sucher A, Lu BC (1997) Fungal galectins, sequence and specificity of two isolectins from *Coprinus cinereus*. J Biol Chem 272:1514–1521.

Coppin E, Debuchy R, Arnaise S, Picard M (1997) Mating types and sexual development in filamentous ascomycetes. Microbiol Mol Biol Rev 61:411–428

Crenshaw RW, Harper SN, Moyer M, Privalle LS (1995) Isolation and characterization of a cDNA clone encoding a lectin gene from *Agaricus bisporus*. Plant Physiol 107:1465–1466

Dahlberg A, Jonsson L, Nylund JE (1997) Species diversity and distribution of biomass above- and below-ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in South Sweden. Can J Bot 75:1323–1335

Danell E, Camacho FJ (1997) Successful cultivation of the golden chanterelle. Nature 385:303

De Groot PWJ, Schaap PJ, Van Griensven LJLD, Visser J (1997) Isolation of developmentally regulated genes from the edible mushroom *Agaricus bisporus*. Microbiology 143:1993–2001

De Roman M, Boa E, Woodward S (2006) Wild-gathered fungi for health and rural livelihoods. Proc Nutr Soc 65:190–197

Debaud JC, Gay G (1987) *In vitro* fruiting under controlled conditions of the ectomycorrhizal fungus *Hebeloma cylindrosporum* associated with *Pinus pinaster*. New Phytol 105:429–435

Debaud JC, Gay G, Bruchet G (1986). Intraspecific variability in an ectomycorrhizal fungus: *Hebeloma cylindrosporum*. I-Preliminary studies on in vitro fruiting spore germination and sexual comportment. In: Gianinazzi-Pearson V, Gianinazzi S, (eds) Physiological and genetical aspects of mycorrhizae. INRA, Paris, pp 581–588

Debaud JC, Gay G, Prevost A, Lei J, Dexheimer J (1988) Ectomycorrhizal ability of genetically different homokaryotic and dikaryotic mycelia of *Hebeloma cylindrosporum*. New Phytol 108:323–328

Douet JP, Castroviejo M, Mabru D, Chevalier G, Dupre C, Bergougnoux F, Ricard JM, Medina B (2004) Rapid molecular typing of *Tuber melanosporum, T. brumale* and *T. indicum* from tree seedlings and canned truffles. Anal Bioanal Chem 379:668–673

Dupré C, Chevalier G, Branlard G (1985) Caractérisation des mycorhizes de différents *Tuber* par l'étude du polymorphisme enzymatique. C.R. 1er Coll. Natl. Sur les technies de purification des protéines, Paris, 1–3 octobre 1984, DIPCINPL public, pp 465–467

Egli S, Peter M, Buser C, Stahel W, Ayer F (2006) Mushroom picking does not impair future harvests - results of a long-term study in Switzerland. Biol Conserv 129:271–276

Eriksson OE (2006) Outline of Ascomycota. Myconet 12:1–82

Ewaze JO, Moore D, Stewart GR (1978) Coordinate regulation of enzymes involved in ornithine metabolism and its relation to sporophore morphogenesis in *Coprinus cinereus*. J Gen Microbiol 107:343–357

Fasolo-Bonfante P, Brunel A (1972) Caryological features in a mycorrhizal fungus: *Tuber melanosporum* Vitt. Allionia 18:5–11

Fassi B, De Vecchi E (1962) Ricerche sulle micorrize ectotrofiche del Pino strobo in vivaio. I. descrizione di alcune forme più diffuse in Piemonte. Allionia 8:133–152

Fassi B, Fontana A (1969) Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum*. II. Sviluppo dei semenzali trapienti e produzione di ascocarpi. Allionia 15:115–120

Ferdman Y, Aviram S, Roth-Bejerano N, Trappe JM, Kagan-Zur V (2005) Phylogenetic studies of *Terfezia pfeilii* and *Choiromyces echinulatus* (Pezizales) support new genera for southern African truffles: Kalaharituber and Eremiomyces. Mycol Res 109:237–245

Fernandez Espinar MT, Labarere J (1997) Cloning and sequencing of the Aa-Pri1 gene specifically expressed during fruiting initiation in the edible mushroom *Agrocybe aegerita*, and analysis of the predicted amino-acid sequence. Curr Genet 32:420–424

Flegg PB, Wood DA (1985) Growth and fruiting. In: Flegg PB, Spencer DM, Wood DA (eds) The biology and technology of the cultivated mushroom. Wiley, Chichester, UK, pp 141–177

Floriani M, Simonini G, Sitta N (2000) *Boletus violaceofuscus*, una specie orientale commercializzata in Italia. Boll Gruppo Micolo G. Bresadola - Nuova Serie 43:4–15

Fogel R, Trappe JM (1978) Fungus consumption (Mycophagy) by small animals. Northwest Sci 52:1–31

Fontana A (1968) Miceli di funghi ipogei in coltura pura. Atti congresso Internazionale sul tartufo, Spoletto 24–25 maggio 1968

Fontana A (1971) Il micelio di *Tuber melanosporum* Vitt. in coltura pura. Allionia 17:19–23

Fontana A, Palenzona M (1969) Sintesi micorrizica di *Tuber albidum* in coltura pura, con *Pinus strobus* e pioppo Euro Americano. Allionia 15:100–104

Frizzi G, Lalli G, Miranda M, Pacioni G (2001) Intraspecific isoenzyme variability in Italian population of the white truffle *Tuber magnatum*. Mycol Res 105:365–369

Gabella S, Abbà S, Duplessis S, Montanini B, Martin F, Bonfante P (2005).Transcript profiling reveals novel marker genes involved in fruit body formation in *Tuber borchii*. Eukaryot Cell 5:1599–1602

Gandeboeuf D, Dupré C, Chevalier G (1994) Différenciation des truffes européennes d'intérêt commercial par analyse des isoenzymes. Acta Bot Gallica 141:455–463

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes- application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118

Giomaro G, Sisti D, Zambonelli A, Amicucci A, Cecchini M, Comandini O, Stocchi V (2002) Comparative study and molecular characterization of ectomycorrhizas in *Tilia americana* and *Quercus pubescens* with *Tuber brumale*. FEMS Microbiol Lett 216:9–14

Giovannetti G, Fontana A (1980–1981). Simbiosi micorrizica di *Tuber macrosporum* Vitt. con alcune Fagales. Allionia 24:13–17

Grimaldi B, De Raaf MA, Filetici P, Ottonello S, Ballario P (2005). Agrobacterium-mediated gene transfer and enhanced green fluorescent protein visualization in the mycorrhizal ascomycete *Tuber borchii*: a first step towards truffle genetics. Curr Genet 48:69–74

Gulden G, Høiland K, Bendiksen K, Brandrud TE, Foss BS, Jenssen HB, Laber D (1992) Macromycetes and air pollution. Mycocoenological studies in three oligotrophic spruce forests in Europe. Bibl Mycol 144:1–81

Gutierrez A, Morte A, Honrubia M (2003) Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorrhiza 13:299–307

Halász K, Bratek Z, Szego D, Rudnoy S, Racz I, Lasztity D, Trappe JM (2005) Tests of species concepts of the small, white, European group of *Tuber* spp. based on morphology and rDNA ITS sequences with special reference to *Tuber rapaeodorum*. Mycol Progr 4:281–290

Hall IR, Wang Y (2002) Truffles and other edible mycorrhizal mushrooms - some new crops for the Southern Hemisphere. Edible mycorrhizal mushrooms and their cultivation. In: Hall IR, Danell E, and Zambonelli A (eds) 2nd International Conference on Edible Mycorrhizal Mushrooms. New Zealand Institute for Crop & Food Research Limited, Christchurch, New Zealand, 3–6 July (2001)

Hall IR, Wang Y, Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438

Hall IR, Lyon AJE., Wang Y, Sinclair L (1998) Ectomycorrhizal fungi with edible fruiting bodies - 2. *Boletus edulis*. Econ Bot 52:44–56

Hamada M (1970) Diaries on Armillaria matsutake. Trans Mycol Soc Jpn 6:81–86

Hansen K, Laessoe T, Pfister DH (2001) Phylogenetics of the Pezizaceae, with an emphasis on Peziza. Mycologia 93:958–990

Hansen K, LoBuglio KF, Pfister DH (2005) Evolutionary relationships of the cup-fungus genus Peziza and Pezizaceae inferred from multiple nuclear genes: RPB2, beta-tubulin, and LSU rDNA. Mol Phylogenet Evol 36:1–23

Hanson AM, Hodge KT, Porter LM (2003) Mycophagy among primates. Mycologist 17:6–10

Helbling A, Bonadies N, Brander KA, Pichler WJ (2002) *Boletus edulis*: a digestion-resistant allergen may be relevant for food allergy. Clin Exp Allergy 32:771–775

Hongo T (1960) The Agaricales of Japan 1–2. Geobot. Acta Phytotax 18:97–112.

Imazeki R, Hongo T (1989) Coloured illustrations of mushrooms of Japan II. Osaka.

Iotti M, Amicucci A, Stocchi V, Zambonelli A (2002) Morphological and molecular characterization of mycelia of some *Tuber* species in pure culture. New Phytol 155:499–505

Iotti M, Barbieri E, Stocchi V, Zambonelli A (2005) Morphological and molecular characterization of mycelia of ectomycorrhizal fungi in pure culture. Fungal Divers 19:51–68

Jahn H, Jahn MA (1986) Konstanz und Fluktuationen der Pilzvegetation in Norra Warleda (Uppland). Beobachtungen auf einem schwedishen Bauernhof 1945–1980. Westfälische Pilzbrief 10–11:352–378

Jansen E, Van Dobben HF (1987) Is decline of *Cantharellus cibarius* in the Netherlands due to air pollution? Ambio 16:211–213

Johnson CN (1996) Interactions between mammals and ectomycorrhizal fungi. Trends Ecol Evol 11: 503–507

Kim HW, Kim BK (1999) Biomedicinal triterpenoids of *Ganoderma lucidum* (Curt.: Fr.) P.Karst (Aphyllophoromycetidae). Int J Med Mushrooms 1:121–138

Kirk PM, Cannon PF, David JC, Stalpers JA (2001) Dictionary of the fungi, 9th edn. CABI, Wallingford, UK

Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol Mol Biol Rev 64:316–353

Kües U, Liu Y (2000) Fruiting body production in Basidiomycetes. Appl Microbiol Biotechnol 54:141–152

Lacourt I, Duplessis S, Abbà S, Bonfante P, Martin F (2002) Isolation and characterization of differentially expressed genes in the mycelium and fruiting body of *Tuber borchii*. Appl Environ Microbiol 68:4574–4582

Lanfranco L, Wyss P, Marzachi C, Bonfante P (1993) DNA probes for identification of the ectomycorrhizal fungus *Tuber magnatum* Pico. FEMS Microbiol Lett 114:245–251

Lanfranco L, Arlorio M, Matteucci A, Bonfante P (1995) Truffles: their life cycle and molecular characterization. In: Stocchi V., Nutti M (eds) Biotechnology of ectomycorrhizae. Molecular approaches. Plenum, New York, pp 139–149

Lannoy G, Estades A (2001) Flore mycologique d'Europe. Vol. 6: Les Bolets. Doc. Myc. Mem. Hors Série 6:p 163

Le Tacon F, Garbaye J, Bouchard D, Chevalier G, Olivier JM, Guimberteau J, Poitou N, Frochot A (1988) Lalonde, Pichey (eds) Field result from ectomycorrhizal inoculation in France. In: Canadian workshop on Mycorrhizal in Forestry pp 51–74

Leonardi M, Paolocci F, Rubini A, Simonini G, Pacioni G (2005) Assessment of inter- and intraspecific variability in the main species of *Boletus edulis* complex by ITS analysis. FEMS Microbiol Lett 243:411–416

Leung GS, Zhang M, Xie WJ, Kwan HS (2000) Identification by RNA fingerprinting of genes differentially expressed during the development of the basidiomycete *Lentinula edodes*. Mol Gen Genet 262:977–990

Lindequist U, Niedermeyer THJ, Julich WD (2005) The pharmacological potential of mushrooms. eCAM 2:285–299

Longato S, Bonfante P (1997) Molecular identification of mycorrhizal fungi by direct amplification of microsatellite regions. Mycol Res 101:425–432

Luis P (2000) Caractérisation de la diversité génétique des populations chez Tuber uncinatum et Tuber melanosporum. DEA de l'université Claude Bernard, Lyon 1 - France

Luoma DL, Trappe JM, Claridge AW, Jacobs KM, Cazares E (2003) Relationships among fungi and small mammals in forested ecosystems. In: Zable CJ, Anthony RG (eds) Mammal community dynamics: management and conservation in the coniferous forests of western North America. Cambridge University Press, Cambridge, pp 343–373

Lurz PWW, South AB (1998) Cached fungi in non-native conifer forests and their importance for red squirrels (*Sciurus vulgaris* L.). J Zool 246:468–471

Lutzoni F, Kauff F, Cox JC, McLaughlin D, Celio G, Dentinger B et al. (2004) Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. Am J Bot 91:1446–1480

Mabru D, Dupré C, Douet JP, Leroy P, Ravel C, Ricard JM, Medina B, Castroviejo M, Chevalier G (2001) Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products: fruiting bodies and mycorrhizal seedlings. Mycorrhiza 11:89–94

Mabru D, Douet JP, Mouton A, Dupre C, Ricard JM, Medina B, Castroviejo M, Chevalier G (2004) PCR-RFLP using a SNP on the mitochondrial Lsu-rDNA as an easy method to differentiate *Tuber melanosporum* (Perigord truffle) and other truffle species in cans. Int J Food Microbiol 94:33–42

Marmeisse R, Guidot A, Gay G, Lambilliotte R, Sentenac H, Combier JP, Melayah D, Fraissinet-Tachet L, Debaud JC (2004) *Hebeloma cylindrosporum* - a model species to study ectomycorrhizal symbiosis from gene to ecosystem. New Phytol 163:481–498

Martin F, Tuskan GA, DiFazio Sp, Lammers P, Newcombe G, Podila GK (2004) Symbiotic sequencing for the *Populus* mesocosm. New Phytol 161:330–334

Maser C, Trappe JM, Nussbaum RA (1978) Fungal-small mammal interrelationship with emphasis on Oregon coniferous forests. Ecology 59:799–809

Maser Z, Maser C, Trappe JM (1985) Food habits of the northern flying squirrel (*Glaucomys sabrinus*) in Oregon. Can J Zool 63:1084–1088

Mello A, Garnero L, Bonfante P (1999) Specific PCR-primers as a reliable tool for the detection of white truffles in mycorrhizal roots. New Phytol 141:511–516

Mello A, Vizzini A, Longato S, Rollo S, Bonfante P, Trappe JM (2000) *Tuber borchii* versus *T. maculatum*: neotype studies and DNA analyses. Mycologia 92:326–333

Mello A, Fontana A, Meotto F, Comandini O, Bonfante P (2001) Molecular and morphological characterization of *Tuber magnatum* mycorrhizas in a long-term survey. Microbiol Res 155:279–284

Mello A, Cantisani A, Vizzini A, Bonfante P (2002) Genetic variability of *Tuber uncinatum* and its relatedness to other black truffles. Environ Microbiol 4:584–594

Mello A, Murat C, Vizzini A, Gavazza V, Bonfante P (2005) *Tuber magnatum* Pico, a species of limited geographical distribution: its genetic diversity inside and outside a truffle ground. Environ Microbiol 7:55–65

Mello A, Murat C, Bonfante P (2006a) Truffles: much more than a prized and local fungal delicacy. FEMS Microbiol Lett 260:1–8

Mello A, Ghignone S, Vizzini A, Sechi C, Ruiu P, Bonfante P (2006b) ITS primers for the identification of marketable Boletus. J Biotechnol 121:318–329

Miyazaki Y, Nakamura M, Babasaki K (2005) Molecular cloning of developmentally specific genes by representational difference analysis during the fruiting body formation in the basidiomycete *Lentinula edodes*. Fungal Genet Biol 42:493–505

Molitoris HP (2002) Pilze in Medizin, Folklore und Religion (Mushrooms in medicine, folklore and religion). Feddes Repert 113:165–182

Montanini B, Betti M, Marquez AJ, Balestrini R, Bonfante P, Ottonello S (2003) Distinctive properties and expression profiles of glutamine synthetase from a plant symbiotic fungus. Biochem J 373:357–368

Montemartini Corte A, Vianello A (1996) Diversi aspetti del micelio di *Boletus edulis* e *B. aereus* e micorrizazione di alcune specie arboree. Micol Ital 3:75–79

Moor DB, P, Nicholas PG, Eugster A (2002) Polymerase chain reaction (PCR) for the detection of king bolete (*Boletus edulis*) and slippery jack (*Suillus luteus*) in food samples. Eur Food Res Technol 214:340–345

Morrissey JP, Dow JM, Mark GL, O'Gara F (2004) Are microbes at the root of a solution to world food production? Rational exploitation of interactions between microbes and plants can help to transform agriculture. EMBO reports 5:922–926.

Mouches C, Duthil P, Poitou N, Delmas J, Bove JM (1981) Caractérisation des espèces truffières par analyse de leurs protéines en gels de polyacrylamide et application de ces techniques à la taxonomie des champignons. Mushroom Sci 11:819–831

Murat C, Diez J, Luis P, Delaruelle C, Dupré C, Chevalier G, Bonfante P, Martin F (2004) Polymorphism at the ribosomal DNA ITS and its relation to postglacial re-colonization routes of the Perigord truffle *Tuber melanosporum*. New Phytol 164:401–411

Murat C, Vizzini A, Bonfante P, Mello A (2005) Morphological and molecular typing of the below-ground fungal community in a natural *Tuber magnatum* truffle-ground. FEMS Microbiol Lett 245:307–313

Murata H, Ohta A, Yamada A, Narimatsu M, Futamura N (2005) Genetic mosaics in the massive persisting rhizosphere colony "shiro" of the ectomycorrhizal basidiomycete *Tricholoma matsutake*. Mycorrhiza 15:505–512

Nelson MA (1996) Mating systems in ascomycetes: a romp in the sac. Trends Genet 12:69–74

Norvell LL (1995) Loving the chanterelle to death? The ten-year Oregon chanterelle project. McIlvainea 12:6–23

Norvell LL, Roger J (1998) The Oregon Cantharellus study project: Pacific golden chanterelle preliminary observations and productivity data 1986–1997. Inoculum 49:40

Norvell LL,Roger J, Lindgren J, Kopecky F (1996) Study 8:Oregon *Cantharellus* study project, 1986–1996. In: Pilz D, Molina R (eds) Managing forest ecosystems to conserve fungus diversity and sustain wild mushroom harvests. Gen. Tech. Rep. PNW-GTR-371. Portland, OR: U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station, pp 69–72

O'Donnell K, Cigelnik E, Weber NS, Trappe JM (1997) Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. Mycologia 89:48–65

Ogawa M (1978) Biology of matsutake mushroom. Tsukiji Shokan, Tokyo

Olivier JM (1991) Champignon comestibles et diversification. Mushroom Inf 4:20–21

Olivier JM (2000) Progress in the cultivation of truffles. Mushroom science XV. Vol. 2. In: Van Griensven LJLD (eds) Proceedings of the 15th International Congress on the Science and Cultivation of Edible Fungi, Balkema, Rotterdam, Netherlands, 15–19 May (2000) Balkema, Rotterdam, Netherlands, pp 937–942

Ospina-Giraldo MD, Collopy PD, Romaine CP, Royse DJ (2000) Classification of sequences expressed during the primordial and basidiome stages of the cultivated mushroom *Agaricus bisporus*. Fungal Genet Biol 29:81–94

Pacioni G, Pomponi G (1991) Genotypic patterns of some Italian species of *Tuber aestivum-Tuber mesentericum* complex. Mycotaxon 42:171–179

Palenzona M (1969) Sintesi micorrizica tra *Tuber aestivum* Vitt., *Tuber brumale* Vitt., *Tuber melanosporum* Vitt. e semenziali di *Corylus avellana* L. Allionia 15:121–131

Palma F, Agostini D, Cerigini E, Polidori E, Stocchi V (2005) Expression and purification of a *Tuber borchii* fruitbody-specific protein, TBF-1, from *Escherichia coli*: generation of polyclonal antibodies. Prep Biochem Biotechnol 35:145–53

Paolocci F, Rubini A, Granetti B, Arcioni S (1999) Rapid molecular approach for a reliable identification of *Tuber* spp. Ectomycorrhizae. FEMS Microbiol Lett 28:23–30

Paolocci F, Rubini A, Riccioni C, Granetti B, Arcioni S (2000) Cloning and characterization of two repeated sequences in the symbiotic fungus *Tuber melanosporum* Vitt. FEMS Microbiol Ecol 34:139–146

Paolocci F, Rubini A, Riccioni C, Topini F, Arcioni S (2004) *Tuber aestivum* and *Tuber uncinatum*: two morphotypes or two species? FEMS Microbiol Lett 235:109–115

Paolocci F, Rubini A, Riccioni C, Arcioni S (2006) Reevaluation of the life cycle of *Tuber magnatum*. Appl Environ Microbiol 72:2390–2393

Pataky JK, Chandler MA (2003) Production of huitlacoche, *Ustilago maydis*: timing inoculation and controlling pollination. Mycologia 95:1261–1270

Percudani R, Trevisi A, Zambonelli A, Ottonello S (1999) Molecular phylogeny of truffles (Pezizales: Terfeziaceae, Tuberaceae) derived from nuclear rDNA sequence analysis. Mol Phylogenet Evol 13:169–180

Peter M, Ayer F, Egli S (2001) Nitrogen addition in Norway spruce stand altered macromycete sporocarp production and below-ground ectomycorrhizal species composition measured by PCR-RFLP analysis of the ribosomal ITS-region. New Phytol 149:311–324

Pierleoni R, Buffalini M, Vallorani L, Guidi C, Zeppa S, Sacconi C, Pucci P, Amoresano A, Casbarra A, Stocchi V (2004) *Tuber borchii* fruiting body: 2-dimensional profile and protein identification. Phytochemistry 65:813–820

Pilz D, Molina R (2002) Commercial harvests of edible mushrooms from the forests of the Pacific Northwest United States: issues, management and monitoring for sustainability. For Ecol Manage 155:3–16

Pöggeler MA (2001) Mating-type genes for classical strain improvements of ascomycetes. Appl Microbiol Biotechnol 56:589–601

Poma A, Colafarina S, Limongi T, Pacioni G (2005) *Tuber borchii* mycelial protoplasts isolation, characterization and functional delivery of liposome content, a new step towards truffles biotechnology. FEMS Microbiol Lett 253:331–337

Poma A, Limongi T, Pacioni G (2006) Current state and perspectives of truffle genetics and sustainable biotechnology. Appl Microbiol Biotechnol 72:437–441

Pyare S, Longland WS (2001) Patterns of ectomycorrhizal-fungi consumption by small mammals in remnant old-growth forests of the Sierra Nevada. J Mamm 82:681–689

Rai RD (1997) Medicinal Mushrooms. In: Rai RD, Dha BL, Verma RN (eds) Advances in mushroom biology and production. National Research Centre Mushrooms, Chambaghat, Solan, pp 355–368

Redhead SA, Norvell LL, Danell E (1997) *Cantharellus formosus* and the Pacific golden chanterelle harvest in western North America. Mycotaxon 65:285–322

Reshetnikov SV, Wasser SP, Tan KK (2001) Higher Basidiomycota as a source of antitumor and immunostimulating polysaccharides. Int J Med Mushrooms 3:361–394

Rogerson CT, Samuels GJ (1994) *Agaricicolous* species of Hypomyces. Mycologia 86:839–866

Rojas C, Mansur E (1995) Ecuador: informaciones generales sobre productos non madereros en Ecuador. Memoria, consulta de expertos sobre productos forestales no madereros para America Latina y el Caribe. FAO Regional Office for Latin America and the Caribbean, Santiago (Chile), pp 208–223

Rubini A, Paolocci F, Granetti B, Arcioni S (1998) Single step molecular characterization of morphologically similar black truffle species. FEMS Microbiol Lett 164:7–12

Rubini A, Paolocci F, Granetti B, Arcioni S (2001) Morphological characterization of molecular-typed *Tuber magnatum* ectomycorrhizae. Mycorrhiza 11:179–185

Rubini A, Paolocci F, Riccioni C, Vendramin GG, Arcioni S (2005) Genetic and phylogeographic structures of the symbiotic fungus *Tuber magnatum*. Appl Environ Microbiol 71:6584–6589

Salerni E, Perini C (2004) Experimental study for increasing productivity of *Boletus edulis* s.l. in Italy. For Ecol Manage 201:161–170

Saltarelli R, Ceccaroli P, Vallorani L, Zambonelli A, Citterio B, Malatesta M, Stocchi V (1998) Biochemical and morphological modifications during the growth of *Tuber borchii* mycelium. Mycol Res 102:403–409

Saupe SJ (2000) Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. Microbiol Mol Biol Rev 64:489–502

Scrase RJ, Elliott TJ (1998) Biology and technology of mushroom culture. In: Wood, B. J. B. (Eds) Microbiology of fermented food, vol 2, 2nd edn. Blackie, London pp 543–584

Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla*, (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. Microb Ecol 47:416–426

Simonini G, Floriani M, Binder M, Besl H (2001) Two close extraeuropean Boletes: *Boletus violaceofuscus* and *Boletus separans*. Micol Veget Mediterranea XVI: 148–170

Singer R (1986) The Agaricales in modern taxonomy 4th edn. Koeltz, Koenigstein

Sirand-Pugnet P, Santos C, Labarere J (2003) The Aa-Pri4 gene, specifically expressed during fruiting initiation in the *Agrocybe aegerita* complex, contains an unusual CT-rich leader intron within the 5' uncoding region. Curr Genet 44:124–131

Sisti D, Zambonelli A, Giomaro G, Rossi I, Ceccaroli P, Citterio B, Benedetti PA, Stocchi V (1998) *In vitro* mycorrhizal synthesis of micropropagated *Tilia platyphyllos* Scop. plantlets with *Tuber borchii* Vittad. mycelium in pure culture. Acta Horticul. 457:379–387

Suz LM, Martin MP, Colinas C (2006) Detection of *Tuber melanosporum* DNA in soil. FEMS Microbiol Lett 254:251–257

Taylor J.W, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS, Fisher MC (2000). Phylogenetic species recognition and species concepts in fungi. Fungal Genet Biol 31:21–32

Taylor RJ (1992) Seasonal changes in the diet of the Tasmanian bettong (*Bettongia gaimardi*) a mycophagous marsupial. J Mamm 73:408–414

Tedersoo L, Hansen K, Perry BA, Kjoller R (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytol 70:581–596

Thiers HD (1984) The secotioid syndrome. Mycologia 76:1–8

Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U et al. (2006) The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313:1596–1604

Unestam T, Stenstrom E (1989) A method for observing and manipulating roots and root associated fungi on plants growing in nonsterile substrates. Scand J For Res 4:51–58.

Urban A, Neuner-Plattner I, Krisai-Greilhuber I, Haselwandter K (2004) Molecular studies on terricolous microfungi reveal novel anamorphs of two Tuber species. Mycol Res 108 (7): 749–758

Urbanelli S, Sallicandro P, De Vito E, Bullini L, Palenzona M, Ferrara AM (1998) Identification of *Tuber* mycorrhizae using multilocus electrophoresis. Mycologia 90:389–395

Villanueva C (1997) 'Huitlacoche' (*Ustilago maydis*) as a food in Mexico. Micologia Neotropica Aplicada 10:73–81

Vizzini A (2005) Il genere *Tuber*: la sua posizione nelle Pezizales (Origine dei taxa ipogei nelle Pezizales). Atti del X Seminario Internazionale sulle Russulales e Boletales e del VI Seminario Internazionale dedicato allo studio dei funghi ipogei, Baselga di Pinè (TN), 2–6 Ottobre (2002). Boll Gruppo Micol Bresadola - Nuova Serie 46:97–153

Wang Y, Hall IR (2004) Edible ectomycorrhizal mushrooms: challenges and achievements. Can J Bot 82:1063–1073

Wang Y, Hall IR, Evans L (1997) Ectomycorrhizal fungi with edible fruiting bodies. 1. *Tricholoma matsutake* and related fungi. Econ Bot 51:311–327

Wang Y, Buchanan P, Hall I (2002) A list of edible ectomycorrhizal mushrooms. In: I.R. Hall, Y. Wang, A. Zambonelli, E. Danell, (eds) Edible ectomycorrhizal mushrooms and their cultivation. Proceedings of the second international conference on edible ectomycorrhizal mushrooms. CD-ROM. Christchurch, New Zealand Institute for Crop and Food Research Limited.

Wang YC (1987) Mycology in ancient China. Mycologist 1:59–61

Wasser S P (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol 60:258–274

Wasser SP, Weis AL (1999) Medicinal properties of substances occurring in Higher Basidiomycetes mushrooms: current perspectives. Int J Med Mushrooms 1:31–62

Weden C, Danell E, Tibell L (2005) Species recognition in the truffle genus *Tuber* – the synonyms *Tuber aestivum* and *Tuber uncinatum*. Environ Microbiol 7:1535–1546.

Wessels JG (1993) Fruiting in the higher fungi. Adv Microbiol Physiol 34:147–202

Wessels JG, Dons JJM, De Vries OMH (1985) Molecular biology of fruit body formation in *Schizophyllum commune*. In: Moore D, Casselton LA, Wood DA, Frankland JC (eds) Developmental biology of higher fungi.. Cambridge University press, Cambridge, pp 485–497

Williams MAJ, Beckett A, Read ND (1985) Ultrastructural aspects of fruit body differentiation in *Flammulina velutipes*. In: Moore D, Casselton LA, Wood DA, Frankland JC (eds) Developmental biology of higher fungi.. Cambridge University Press, Cambridge, pp 429–450

Yamada M, Sakuraba S, Shibata K, Inatomi S, Okazaki M, Shimosaka M (2005) Cloning and characterization of a gene coding for a hydrophobin, Fv-hyd1, specifically expressed during fruiting body development in the basidiomycete *Flammulina velutipes*. Appl Microbiol Biotechnol 67:240–246

Yamanaka K (1997) Production of cultivated edible mushrooms. Food Rev Int 13:327–333

Zaidman BZ, Yassin M, Mahajna J, Wasser SP (2005) Medicinal mushroom modulators of molecular targets as cancer therapeutics. Appl Microbiol Biotechnol 67:453–468

Zeppa S, Guescini M, Potenza L, Agostini D, Polidori E, Stocchi V (2000) Analysis of gene expression in the vegetative and fructification phases of the white truffle, *Tuber borchii* Vittad., by mRNA differential display. Biotechnol Lett 22:307–312

Zhao J, Kwan HS (1999) Characterization, Molecular Cloning, and Differential Expression Analysis of Laccase Genes from the Edible Mushroom *Lentinula edodes*. Appl Environ Microbiol 65:114908–114913

Zhou S, Gao Y (2002) The immunomodulating effects of *Ganoderma lucidum* (Curt.:Fr.) P.Karst (LingZhi, Reishi Mushroom) (Aphylloromycetidae). Int J Med Mushrooms 4:1–11

Zhou Z, Miwa M, Matsuda Y, Hogetsu T (2001) Spatial distribution of the subterranean mycelia and ectomycorrhizae of *Suillus grevillei* genets. J Plant Res 114:179–185

Arbuscular Mycorrhiza in Physiological and Morphological Adaptations of Mediterranean Plants

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1 Introduction

From the physiological and morphological point of view, vascular plants can be considered to be composed of two distinct parts: the aboveground superstructure which is autotrophic and visible, and the belowground substructure which is heterotrophic and mostly invisible. Though more attention has been given to the superstructure, the substructure is equally important for plant and ecosystem functioning, and the two have general functions in common (Fitter 1991). Both parts are structured to enable resource capture. They are made up of organs specialized for resource acquisition, leaves above ground and the distal parts of roots below ground, and of the axes that connect and support these sites of resource acquisition within individual plants. Both also contain structures devoted to mechanical support, resources storage, and reproduction. Both exhibit a vertical structure that responds to resources with a strong vertical dimension – light above ground, nutrients and water below ground – so there ought to be strong communication that alters growth patterns and creates predictable relationships between the two parts.

Although not always evident, plants are not islands. They establish strong relationships with other organisms, especially at the root level where they support a unique modified microbial community. Microorganisms directly colonize root surfaces and the roots themselves, at the same time creating and exploiting additional unique environments in the rhizosphere. In essence, the microorganisms in the rhizosphere provide the critical link between plants, which require inorganic nutrients, and the environment which contains the nutrients, but often in inaccessible forms or locations. This constitutes the rationale for explaining the uniqueness of mycorrhizal associations, and in particular of arbuscular mycorrhizal fungi (AMF).

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This group of fungi originated between 353 and 462 million years ago and the arbuscular mycorrhiza (AM) symbiosis is probably similarly ancient and important in the colonization of land by vascular plants (Pirozynski and Dalpé 1989; Remy et al. 1994; Smith and Read 1997).

AMF form mycorrhizal symbioses with two thirds of all plant species, and are believed to be wholly dependent on the plant partner for their carbon supply, possessing no degradative capacity. There is excellent evidence that external hyphae of AMF actively absorb non-mobile nutrients (P, Zn, Cu) from soil and translocate them rapidly into the plant roots. AMF and plant roots share the same habitat, the soil, and for both the primary source of nutrients must be the soil solution, in equilibrium with the so-called soil labile fraction. However, hyphae are able to penetrate soil pores inaccessible to roots and may also be able to compete efficiently with soil-inhabiting microorganisms for recently mineralized nutrients.

AMF are known mainly for their involvement in P absorption by plants, but they are also associated with other cell functions. In fact, changes in host plants induced by AMF associations have many functional responses besides increased P uptake. Mycorrhizal plants contain a modified endogenous balance of growth regulators (Berta et al. 1993). Increased cytokinin and decrease abscisic acid production has been observed in host leaves and roots. These changes interfere with cell size, cell cycle, biomass allocation, root and shoot morphology and soil–plant–atmosphere water relations, and increase tolerance to biotic and abiotic stresses (Johansen et al. 1992).

Given such a broad spectrum of plant responses to AM colonization, it is reasonable to ask if there are any true systemic effects of this particular symbiosis on host plants? It has been shown that mycorrhizal plants show different gene expression patterns in leaf and root tissues (Taylor and Harrier 2003), which can provide insight to the mechanisms by which the AM symbioses influence the host plant metabolism and physiology, and raises questions about the existence of biochemical pathways or changes in metabolic activities triggered by AMF in the shoots and /or roots of AM plants.

In this chapter, we would like to show that the responsiveness of plants to AM symbiosis in slow-growing wild species is dependent on soil AMF communities. Results suggest that plants' profit from the symbiosis is dependent on physiological changes on plant roots and shoots leading to increased fluxes of carbon from the shoots to the roots and of N in the opposite direction. It seems that, under low resource availability, (1) root and shoot physiological adaptations occur in order to maximize resource acquisition capacity, and (2) morphological adaptations increase the areas of resource acquisition, improving plant responsiveness to AM symbiosis.

Several results will be presented in order to highlight the importance of AM symbiosis to N acquisition by Mediterranean-type plants, which may well constitute an adaptation to environments where water availability is reduced and soil is highly heterogeneous (Cruz et al. 2004).

2 Physiological and Morphological Changes in Shoots due to AM Symbiosis

Since plants are composed of two parts, both specialized in resource acquisition, it is reasonable to consider that the relative development of each part is dependent on the type of resource that is most limiting to plant growth (Lambers et al. 1998; Martins-Loução and Cruz 1999; Martins-Loução and Lips 2000). If one of the advantages of AM symbiosis is an improvement in nutrient acquisition, it would be expected to have effects on the size and structure of the shoot and consequently on the root–shoot ratio of the plant. In fact, many studies have claimed improvements in biomass accumulation due to AM symbiosis based only on the comparison of shoot biomass or size.

Plants with lower root–shoot ratios have higher photosynthetic potential and lesser root capacity to support a potentially larger shoot demand than those with higher root–shoot ratios. Thus, they may enhance uptake of minerals through high mycorrhizal development. Baylis (1975) also postulated that plants with a low root–shoot ratio are more dependent on extraradical AM hyphae for extraction of minerals from the soil.

Although the increased allocation of biomass to leaves in AM plants is not universal, it has been accepted as a strong indication of a beneficial AM colonization. Research on the primary metabolism of the plant has also been addressed in studies investigating changes in photosynthetic rates and carbon assimilation and allocation in AM plants (Wright and Upadhyaya 1998; Wright et al. 1998). It has been suggested that the symbiosis can increase photosynthesis through morphological adaptation such as increases in specific leaf area (Harris and Paul 1987), leaf water potential and sugar content (Ruiz-Lozano 2003; Caravaca et al. 2005). AM symbiosis has been reported to induce changes in the phytohormonal balance in host plants (Blee and Anderson 1998; Fitze et al. 2005) but there is only limited evidence of such effects in the shoots of AM plants. However, there is a need for further research to elucidate the mechanisms by which AMF affect plant metabolism in shoots (Toussaint 2007).

More recently, the idea has developed that the mycorrhizal association is not specific, since a plant root can be colonized by several species of AM fungi at the same time, but that the outputs of the association are dependent on the species involved. This suggests that different hosts are affected in different ways by fungus species alone or in combination. Not all plants benefit to the same extent from AM symbiosis, and among those that do, the extent of the benefit depends upon the fungus community present in the root medium (Jakobsen et al. 2002). Several factors may increase mycorrhizal responsiveness of autotrophic plants. Important characteristics related with the fungus species are: rates of colonization; growth and extension in the soil; and its capacity for nutrient uptake and translocation. On the other hand, several characteristics of the plant partner are also important: root to shoot ratio; root branching; root diameter; nutrient uptake capacity; and the capacity

of carbon delivery to hyphae root interface. Smith et al. (2003) showed that the biomass and phosphorus gain of mycorrhizal plants as well as the phosphorus acquired though the mycorrhiza is dependent on the plant and fungi species involved in the symbiosis.

Since many of the systemic effects of the AM symbiosis are unknown, it is of interest to compare the effects of root colonization by distinct AM communities on the physiological and morphological characteristics of the shoot.

A study comparing physiological characteristics of shoots of two woody Mediterranean plant species grown in the presence of their native soil AMF communities and in inoculated soil (obtained after the inoculation of sterile soil with *Glomus intraradices* produced by Premier Tech, and addition of the bacterial inoculum) clearly showed that the AMF community strongly affected plant responsiveness (Correia 2006). Both plant species accumulated more biomass when soil was inoculated with *Glomus intraradices* than when in the presence of the native soil AMF community (Fig. 1).

Since no clear differences were observed between P concentrations in the leaves of the plants grown with the distinct AMF communities (Correia 2006), the results imply that increased biomass accumulation was related with greater increases in

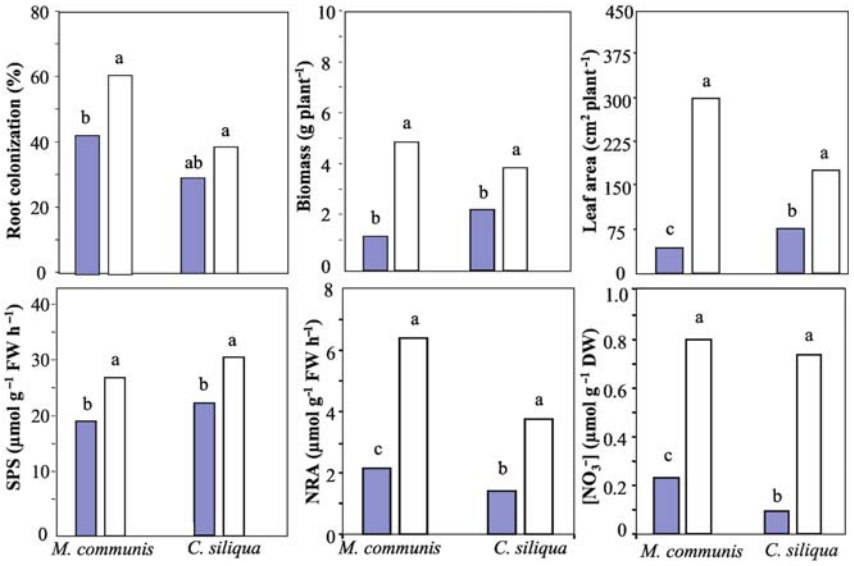

Fig. 1 Percentage of root colonization, shoot biomass accumulation on a dry weight basis, leaf area, leaf sucrose phosphate synthetase (*SPS*) acitivity, leaf nitrate reductase acitivity (*NRA*) and leaf nitrate concentration were determined in plants belonging to two species, *Ceratonia siliqua* (carob) and *Myrtus communis* (myrtle). Plants were grown in 6-l pots under greenhouse conditions in Portugal (38°27'34"N, 9°0'20"W) for 6 months. The soil chemical composition was equivalent for all the treatments. Two AMF communities were studied: native (*dark bars*), and inoculated, obtained after the inoculation of sterile soil with *Glomus intraradices* (produced by Premier Tech) and the respective soil bacterial community *(open bars). Columns* referring to the same species followed by the same *letters* are not significantly different by Fisher's LSD test at $P = 0.05$

total plant leaf area, leaf nitrate reductase activity and leaf nitrate content (Fig. 1), suggesting the involvement of the AM symbiosis in nitrogen acquisition, assimilation and partitioning between root and shoot. It is of particular interest that the fungal community able to promote shoot biomass accumulation also stimulated nitrate flux and nitrate reductase activity in the shoots (Fig. 1), suggesting the involvement of the AM symbiosis in nitrogen acquisition and partitioning between root and shoot. Several researchers (Bago et al. 1996; Kaldorf et al. 1998) showed increased nitrate reductase activity in both shoots and roots of AM plants, and concluded that the enhancement was brought about by a relief of phosphate stress. On the other hand, nitrate transport from roots to shoots is under the control of hormonal balance, and is promoted by high levels of cytokinins, which have been reported to increase under AM colonization. Regardless of the process by which nitrate is acquired by the roots, its reduction to ammonium and subsequent incorporation into organic compounds in the shoots may be a way to obtain organic nitrogen with lower carbon costs. Nitrate assimilation in leaves may be a photosynthetic process in which reduced ferredoxin from photosystem I functions as an electron donor for nitrate reduction (Flores et al. 2005).

The differences in shoot morphology imposed by AM symbiosis are not only related to root–shoot biomass partition (Auge 2001; Majdi et al. 2001). In an experiment comparing two levels of nutrient addition (high and low) and AM symbiosis (Cruz et al. 2004), AM plants grown with low levels of nutrients were observed to have more expanded leaves and lower specific leaf weight (SLW) (Fig. 2). A decrease in SLW indicates a decrease in biomass accumulation per unit leaf area, probably reflecting lower accumulation of assimilation products (starch, amino acids) or secondary compounds (Lambers et al. 1998). However, it also represents a modulation of leaf area per unit biomass invested in leaves that can be mediated by changes in leaf anatomy. Thick leaves with a high SLW may have extra layers of palisade cells, more chloroplasts and photosynthetic enzymes and thereby higher photosynthetic capacity per unit leaf area. However the increment in the photosynthetic capacity comes at a cost of lower light capture per unit biomass. Consequently, when light is a limiting factor, leaves with low specific leaf weight may contribute relatively more to plant growth (Poorter 2001).

It is widely accepted that AM mycorrhiza improve phosphorous acquisition by the plant. Several studies have shown that AM also improve nitrogen acquisition (Ames et al. 1983; Hawkins et al. 2000; Johansen et al. 1992; Govindarajulu et al. 2005), though this is more controversial. In an experiment performed with *Ceratonia siliqua* L. (a tree characteristic of Mediterranean climates) the gain in biomass due to the AM symbiosis was observed to be correlated with the shoot gain of both P and N, but a better correlation was obtained for N (Fig. 3). Several field and pot experiments have shown that leaf nitrogen discrimination ($\delta^{15}N$) differs between AM-colonized and noncolonized plants (Martins-Loução and Cruz 1999). Mycorrhizal associations have been reported to discriminate against the heavier isotope during the transfer of N from the fungus to the plant host (Handley et al. 1999; Hobbie et al. 2000). This fractionation causes plant tissues to become depleted in ^{15}N (i.e., they have negative $\delta^{15}N$ values). Spriggs et al. (2003) made a

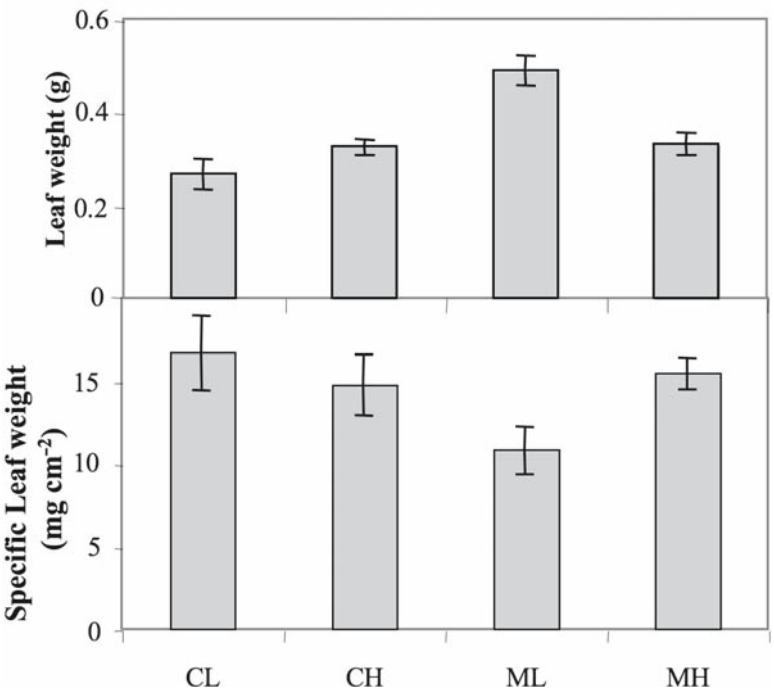

Fig. 2 Leaf weight and specific leaf weight of 3-month-old carob seedlings (non-mycorrhizal or mycorrhizal) grown with low or high nutrient supply to the root medium. Plants grown with low nutrients received a balanced nutrient solution (N as NO_3^- 0.611 g l^{-1}, P as PO_4^{3-} 0.031 g l^{-1} and K^+ at 0.236 g l^{-1}) with an extensive micronutrient supplement (Hewitt, 1966). Plants from high nutrient treatments received a supplement of 300, 900, 480 and 340 mg $plant^{-1}$of P, N, K and Mg, respectively. In total, each plant (one per pot) received 1-51 of nutrient solution. The AM inoculant was an isolate of *Glomus intraradices* (Smith & Schenck) multiplied by pot culture with maize.. A non-AM inoculum was produced by growing maize in the same soil mixture after sterilization and without propagules. Maize plants were allowed to grow for 6 weeks. The roots were cut in small segments and mixed with the soil. *Columns* represent mean values (n = 10). *Columns* followed by the same *letters* within *panel* are not significantly different by Fisher's LSD test at P = 0.05

survey of leaf ∂^{15}N values of plants belonging to the fynbos ecosystem (South Africa) and found that AM functional groups presented significantly lower values than nonmycorrhizal functional groups, concluding that mycorrhizal associations could be the cause of the negative foliar δ^{15}N values. However, even within a mycorrhizal functional group there is a high variability in plant δ^{15}N values. Plant rooting depth, seasonality of N uptake, mobilization of stored N and discrimination amongst ^{15}N along transport between plant parts all cause variability in δ^{15}N values, and complicate interpretation of the results. Consistent results for δ^{15}N values and mycorrhizal functional groups were obtained when leaves were collected at the same time of the year (spring), plants were grown in the same climate conditions (Mediterranean), and leaves were at the same stage of development (young full expanded leaves) (Table 1).

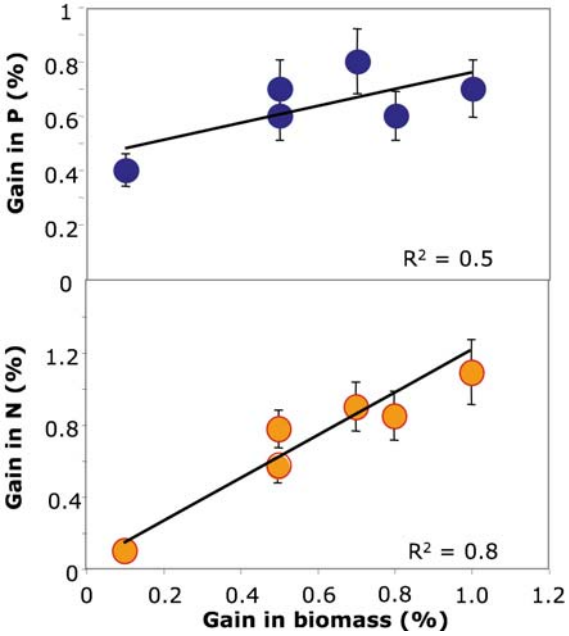

Fig. 3 Gain in shoot phosphorous (*P*) and nitrogen (*N*) concentrations as a function of the gain in shoot biomass per plant of carob seedlings (*Ceratonia siliqua* L. cv. Mulata). Seedlings were grown for 1 year under field conditions after which they were submitted to the distinct treatments. P gain was calculated in plants receiving fixed amounts of P and N additions in order to have a final concentration of N in the soil of 0.02, 0.15, 0.30, 0.60, 0.90 and 1.2%. N gain was calculated in plants receiving a fixed amount of N and P additions in order to have a final concentration of P in the soil of 35, 70, 140, 200, 250 and 320 ppm. Roots were inoculated or not with an isolate of *Glomus intraradices*. Results refer to plants with root colonization >15%. Gain was calculated as (Myc–nonMyc)/non-Myc

Table 1 Foliar ∂^{15}N values of trees and shrubs from Mediterranean-type ecosystems. Leaves were collected in the spring and correspond to the youngest fully developed leaf of the stem. Results represent means (n = 7–10) ± SD

Plant species	Mycorriza	∂^{15}N
Olea europaea L.	AM	−2.3 ± 0.3
Ceratonia siliqua L.	AM	−4.4 ± 0.4
Pistacia lentiscus L.	AM	−2.5 ± 0.5
Myrtus sp. L.	AM	−2.8 ± 0.8
Erica arborea L.	Ericoid	−1.5 ± 0.7

When slow-growing plant species typical of low-nutrient ecosystems are grown in the presence of increased nutrient levels (Table 2), their degree of AM colonization decreases, leading to changes in the parameters associated with mycorrhiza responsiveness.

Table 2 Percentage of root colonization, foliar ∂^{15}N values and N:P ratios of 3-month-old carob seedlings (non-mycorrhizal or mycorrhizal) grown with low or high nutrient supply to the root medium. Plants grown with low nutrients received a balanced nutrient (N as NO_3^- 0.611 g l^{-1}, P as PO_4^{3-} 0.031 g l^{-1} and K^+ at 0.236 g l^{-1}) with an extensive micronutrient supplement (Hewitt 1966). Plants from high nutrient treatments received a supplement of 300, 900, 480 and 340 mg $plant^{-1}$ of P, N, K and Mg, respectively. In total, each plant (one per pot) received 1.5 l of nutrient solution. The AM inoculant was an isolate of *Glomus intraradices* (Smith & Schenck) multiplied by pot culture with maize. A non-AM inoculum was produced by growing maize in the same soil mixture after sterilisation and without propagules. Maize plants were allowed to grow for 6 weeks. The roots were cut in small segments and mixed with the soil. Numbers represent mean values (n = 10) ± SD

	NML	NMH	ML	MH
Root colonization (%)	0.9 ± 0.5	0.8 ± 0.6	25.0 ± 3.5	10.2 ± 1.5
Leaf ∂^{15}N	1.0 ± 0.3	0.9 ± 0.5	−2.4 ± 0.4	0.12 ± 0.10
N:P	15.3 ± 0.2	14.5 ± 2.1	20.1 ± 2.2	15.8 ± 1.4

NML Nonmycorrhizal and low nutrients, *NMH* nonmycorrhizal and high nutrients, *ML* mycorrhizal and low nutrients, *MH* mycorrhizal and high nutrients

It is evident from Table 2 that AM colonization only has an impact on plant development under low nutrient availability (Fig. 1). Under these circumstances, leaves' δ^{15}N values became negative, while those of the non-AM plants did not, in agreement with: (1) the hypothesis that AM fungi are important in nitrogen acquisition by the plant, and (2) that there is a discrimination of the heaviest isotope in the processes between nitrogen acquisition by the extra-root mycelium and its transference to the root. Further research is needed in this area in order to determine under which conditions leaf δ^{15}N can be used as an indicator of plant responsiveness to AM symbiosis.

If AM symbiosis can affect the acquisition of N and P, how does it affect the N:P ratio of the plant material? Biomass N:P ratio reflects the balance between uptake and losses of the nutrients as well as internal nutrient allocation and translocation, and is not directly influenced by the plant carbon economy (Eckstein and Karlsson 2001). The average N:P ratio of plants at their natural locations is 12–13 (Chiou et al. 2005). However, N:P ratios of individual plants can vary 50-fold in response to N and P supply, reflecting variation in nitrogen and/or phosphorous concentrations (Gonçalves 2000; Gonçalves and Martins-Loução 1997). Variations in N:P ratios of graminoid species are primarily determined by variation in P because their N concentration is relatively stable. Variation in the concentration in nitrogen is often more important in determining the N:P ratios of woody plants, bryophytes or lichens. Thus, it seems that AM symbiosis is able to increase the N:P ratio of shoots.

In the AM symbiosis, the fungus receives all of its carbon from the plant. This may increase the strength of the root sink for carbon, corresponding, depending on the species involved, to 4–26% of the carbon fixed by photosynthesis (Black et al. 2000). From an ecological perspective, it would be interesting to assess the relation between nitrogen acquisition via AMF and AM plant photosynthetic rates, since nitrogen is fundamental for chlorophyll content, leaf growth and photosynthesis.

At the physiological level, leaves have some regulatory sites in the chloroplast for starch and sucrose biosynthesis: (1) the triose-P/Pi antiporter, and (2) sucrose phosphate synthase (SPS). The triose-P/Pi translocator, located on the inner membrane of the chloroplast, indirectly controls the triose-P conversion to starch and sucrose synthesis. The triose-P is exported from the chloroplast if there is Pi available to be imported. Under P limitation, plants have been shown to have low P concentrations in the cytosol, which affects the export of triose-P, and starch accumulates in the chloroplast – a common situation associated with low photosynthetic activities. In addition, SPS is a key regulatory enzyme inhibited by Pi. AM plants tend to have higher shoot biomass, SPS activity (Fig. 1) and N:P ratios (Table 2), which could reflect enhanced photosynthetic activities associated with plant responsiveness to AM symbiosis. These regulatory processes show a possible route for the modulation between nutrient acquisition and photosynthetic activity, but further evidence is necessary.

Altogether the results show that AMF symbiosis can (depending on the composition of the fungi community) impose physiological and morphological changes in the shoots of AM slow-growing sclerophyllous species adapted to low nutrient availability, particularly N and P. The results highlight the importance of the N status of the shoot in plant responsiveness to AMF colonization.

3 Physiologial and Morphological Changes in Roots due to AM Symbiosis

Plant foraging for heterogeneously distributed soil resources demonstrates morphological root plasticity, which can be defined by increased root length or mass (i.e., root proliferation), in response to localised nutrient enrichment. Plants may also exploit nutrients by physiological root plasticity which involves a disproportionate increase in the rate of nutrient absorption per unit tissue mass or length when a zone of high nutrient concentration is encountered. This mechanism may be particularly important when soil resources are temporally and spatially too unpredictable to allow effective exploitation by morphological root plasticity, and nutrient pulses are of insufficient magnitude or duration to offset foraging costs associated with morphological root plasticity (Derner and Briske 1999). The relative expression of physiological and morphological root plasticity is probably influenced by growth form, life history strategy and habitat characteristics of individual species. In this section, we would like to show that AM symbiosis may affect both types of root plasticity in a way that is dependent on the soil mycorrhizal community.

Changes in the physiological activity of certain enzymes involved in the root nitrogen metabolism due to AM symbiosis were studied in sclerophyllous plants (*Myrtus communis*, myrtle, and *Ceratonia siliqua*, carob) in an experiment where the effects of two fungi communities were assessed: native – from native soils, and inoculated – obtained after the inoculation of sterile soil with *Glomus intraradices* (produced by Premier Tech,) and reposition of the original bacterial inoculum

(Correia 2006). The magnitudes of the effects of the two fungi communities on the enzymatic activities and metabolite concentrations assessed were distinct. In general, the inoculation of soil with *Glomus intraradices* had stronger effects on the physiology of the AM root of both plants (Fig. 2), in particular in glutamine synthetase (GS), arginase and urease activities of the AM roots. Arginase and urease are two key enzymes in the transference of nitrogen from the mycelium into the plant root in AM symbiosis (Bago et al. 2001; Cruz et al. 2007). According to the model proposed by Bago et al. (2001), the nitrogen is taken up by the extraroot mycelium in the form of nitrate or ammonium, and incorporated in organic compounds by glutamine synthetase. The glutamine produced is fed into the anabolic arm of the urea cycle, leading to the synthesis of arginine, which is loaded into the vacuoles. It is then transported along the hyphae into the intra-root mycelium, where arginine moves out of the vacuole and is loaded into the anabolic arm of the urea cycle in order to be degraded, leading to an increased concentration of urea. In the presence of active urease, the urea can be transformed into ammonium and carbon dioxide. Finally, nitrogen is transferred to the plant root in the form of ammonium. Several key assumptions of the model (Bago et al. 2001) have been proved (Jin et al. 2005; Govindarajulu et al. 2005; Cruz et al. 2007) using AM root organ cultures, and its potential contribution to the nitrogen transport from the fungi to the plant has been estimated to be 3 nmol N mg^{-1} FWh^{-1}. However, the ecological significance of the model under distinct nitrogen availabilities to the fungi and to the root remains to be assessed.

The observed increment of arginase and urease activities in AM roots (Fig. 4) may be considered indicative of the importance of AMF to nitrogen acquisition and transference to the plant as ammonium. However, the distinct effects of AM communities on AM root arginase and urease activities seem to indicate that distinct AMF communities may contribute differently to the improvement of nitrogen acquisition through root physiological plasticity.

Although arginase and urease activities are putatively located in the intraroot mycelium, they might imply an increase in root metabolic activity since carbon transfer from root into the hyphae and ammonium metabolization in the root must increase.

Root carbohydrate pools are substantially altered in AM compared with non-AM plants. For example, starch levels are lower or even absent in arbuscular-containing cortical cells (Bago et al. 2000).

The use of organelle-targeted green fluorescent protein and the complementary results regarding transcript and metabolite levels provided a first overview of structural and metabolic changes of cell organelles during colonization by AM fungi. They showed an increase in the expression of a hexose transporter gene which is probably involved in uptake in cortical cells near intraradical hyphae (Harrison 2005), while changes in the expression of invertase in cells containing arbuscules have also been observed (Schaarschmidt 2006). Respiration in AM roots is also higher than in non-AM roots (Graham et al. 1997; Graham and Abbott 2000). These observations are consistent with the transfer of carbohydrate to the fungus and with AM roots being a stronger sink for photosynthates than non-AM

ones (Douds et al. 2000). The strength of the carbon sink imposed on AM roots is also reinforced by the transfer of ammonium to the roots, since due to the central role of ammonium in plant metabolism, plants try to keep their ammonium cytoplasmatic levels low (Cliquet and Stewart 1993; Britto and Kronzucker 2002). The majority of the ammonium acquired by the plant is therefore integrated into amino acids, through the activity of glutamine synthetase in the root. This implies a coordination in the changes of arginase, urease and glutamine synthetase (GS) activities as observed (Fig. 4).

It is believed that the acidification of the compartment between the symbionts during mycorrhizal colonization is of pivotal importance for nutrient transfer because it energizes several secondary transport systems. Several studies have suggested that the H^+-ATPase is responsible by pumping protons across the periarbuscular membrane generating such acidic compartment (Gianinazzi-Pearson 1991). At the molecular level, the regulation of H^+-ATPase genes by AM has been extensively demonstrated (Gianinazzi-Pearson 2000; Ferrol et al. 2002). On the other hand, most experiments at the biochemical level using microsomal membranes have showed an activation of this pump corresponding to the induction found at molecular level (Paszkowski 2006).

A massive proliferation of plastids and mitochondria has also been observed in colonized root cortical cells (Lohse et al. 2005). The prominent position of the

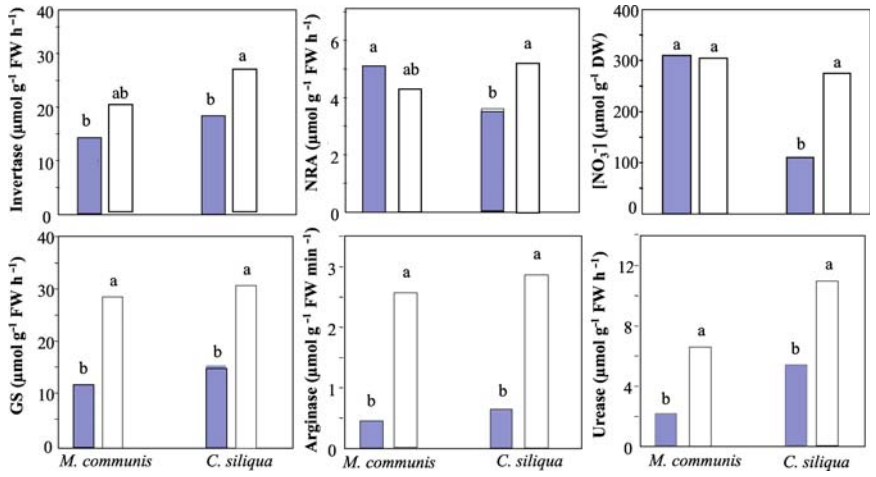
Invertase (µmol g-1 FW h-1)
NRA (µmol g-1 FW h-1)
[NO3-] (µmol g-1 DW)
GS (µmol g-1 FW h-1)
Arginase (µmol g-1 FW min-1)
Urease (µmol g-1 FW h-1)
M. communis
C. siliqua

Fig. 4 Nitrate concentration and enzymatic activities were determined in roots belonging to two species, *Ceratonia siliqua* (carob) and *Myrtus communis* (myrtle). Plants were grown in 6-l pots under greenhouse conditions in Portugal (38°27'34"N, 9°0'20"W) for 6 months. The enzymatic activities determined were: inveratase, nitrate reductase activity (NRA), glutamine synthetase (GS), arginase and urease. The soil chemical composition was equivalent for all the treatments. Two AMF communities were studied: native (*dark bars*), and inoculated, obtained after the inoculation of sterile soil with *Glomus intraradices* (produced by Premier Tech) and the respective soil bacterial community *(open bars). Columns* referring to the same species followed by the same *letters* are not significantly different by Fisher's LSD test at $P = 0.05$

biosynthetic pathways leading to asparagines and aspartate in AM roots reflects the increased demand for protein biosynthesis and the ability of AM symbiosis to provide nitrogen to the host plant.

4 Root Morphology

Until a few years ago, roots were studied in terms of fresh and dry weight, total root length or root to shoot ratio, parameters which are usually unable to reveal differences between AM and non-AM systems. Recently, more accurate analysis based on morphometric analysis of the root apparatus has revealed more subtle differences due to AM symbiosis. Plasticity of the AM root system has been demonstrated across a broad range of plant species in herbaceous as well as in tree plants, and mono and dicotyledons at a number of levels, from gross alterations in biomass partitioning between roots and shoots to altered root morphology and architecture (Fitter 1986; Robinson et al. 2003). These responses are considered adaptive with respect to the maintenance of a functional equilibrium between carbon and mineral acquisition. However, a clear link relating root architecture and mycorrhizal colonisation to the functional aspects of nutrient acquisition is still missing. For Berta et al. (1990, 1993), the activity of the apical meristem defines root development and architecture. Although the AM fungus does not penetrate the meristem, it can regulate the meristem activity simply by acting as a strong sink of photosynthesized carbon.

One of the first ideas was to look for a parameter able to differentiate between AM and non-AM roots. Special attention was paid to differentiating roots based on their pigmentation, to identify possible relations between pigmentation and root physiological activity. In a study of the roots of some mycorrhizal legumes. Jones (1924) discovered a characteristic yellow or greenish-yellow discoloration of these roots, limited to the cell sap of the cortical cells outside the endodermis, which is caused by mycorradicin. The occurrence of this compound correlates well with the yellow coloration of the roots of some plants. However, trace amounts of myxorradicin were also found in nonmycorrhizal roots (Schliemann et al. 2006; Strack and Fester 2006), and the idea of using it as an indicator of AM colonization was abandoned.

An attempt to identify possible relations between pigmentation and root physiological activity was made by differentiating roots based on their pigmentation (Cruz et al. 2004). The onset of browning generally indicates decreased root physiological functionality (López et al. 2001; Comas et al. 2000). Color images of plant root systems were taken and analyzed with Winrhizo (Regents®, Canada) software and three root zones were defined according to the Munsell (1975) soil color chart: the root apex corresponding to the white tips 10YR 7/1-8/4; the elongation zone corresponding to the yellow parts of the root 10YR 3/3-8/7; and the heavily pigmented zone corresponding to darker areas. The percentage of root colonization was shown to be higher in the lighter (30–36%) than in the darker root parts (5.6%).

AM colonization had the effect of increasing the relative percentage of the lighter in relation to the darker areas (Cruz et al 2004). Since AM colonization was affected by nutrient availability, its effect was only significant when plants were grown under low nutrient availability, and consisted of maintaining a high nutrient uptake capacity in conditions of low resource availability (Figs. 5 and 6). The dissection and analyses of the three root sections for their concentrations of 12 elements (N, P, Na, Ca, K. Mg, S, Fe, Cu, Mn, Bo and Zn) showed clear patterns of concentrations on a root dry weight basis, however there were more similarities between the brown and yellow root sections than that between these and the white sections (Fig. 5). Although care is needed when relating concentrations with physiological activities, such strong differences in so many elements must be related with the function of the distinct root parts. These results are in agreement with several others showing that P, K, Ca, Mg and Cu concentrations are higher in AM-colonized than in noncolonized plants (Li et al. 1991, 2003; George et al. 1995). They also highlight that studies of metabolic and physiological changes in roots should be performed in selected parts of the roots. Measurements referring to whole root systems, containing relatively few numbers of colonized cells of various symbiotic stages may underestimate the actual changes in more specific root areas.

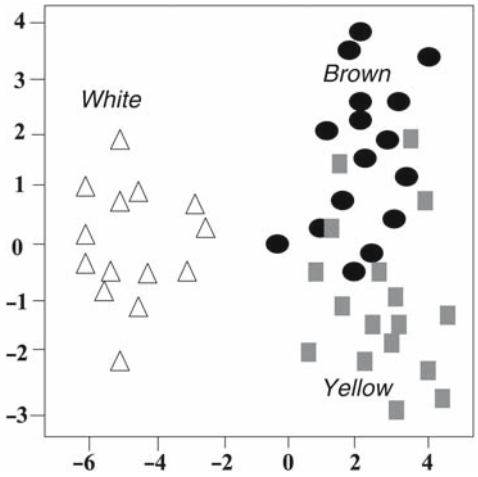

Fig. 5 Canonical variate analysis of 12 element concentrations in carob roots according to root colour (white, yellow and brown). Plants of carob (non-mycorrhizal or mycorrhizal) were grown for 3 months with low or high nutrient supply to the root medium. Plants grown with low nutrients received a balanced nutrient solution ((N as NO_3^- 0.611 g l^{-1}, P as PO_4^{3-} 0.031 g l^{-1} and K^+ at 0.236 g l^{-1}) with an extensive micronutrient supplement (Hewitt 1966). Plants from high nutrient treatments received a supplement of 300, 900, 480 and 340 mg $plant^{-1}$of P, N, K and Mg, respectively. In total, each plant (one per pot) received 1.5 l of nutrient solution. The AM inoculant was an isolate of *Glomus intraradices* (Smith & Schenck) multiplied by pot culture with maize. A non-AM inoculum was produced by growing maize in the same soil mixture after sterilization and without propagules. Maize plants were allowed to grow for 6 weeks. The roots were cut in small segments and mixed with the soil. *Columns* represent mean values (n = 10). *Columns* followed by the same *letters* within *panel* are not significantly different by Fisher's LSD test at P = 0.05

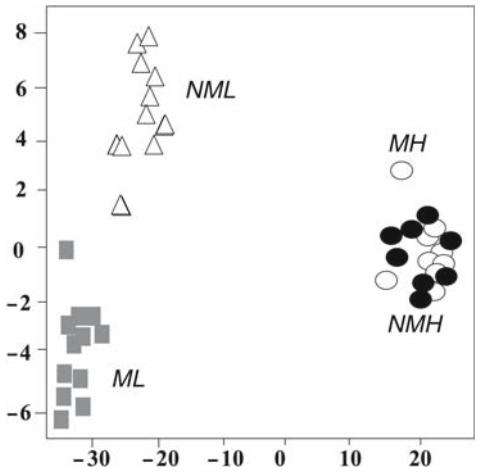

Fig. 6 Canonical variate analysis of 12 element concentrations in carob roots according plant treatment. *NML* Nonmycorrhizal and low nutrients, *NMH* nonmycorrhizal and high nutrients, *ML* mycorrhizal and low nutrients, *MH* mycorrhizal and high nutrients. Plants were grown for 3 months with low or high nutrient supply to the root medium. Plants grown with low nutrients received a balanced nutrient solution (N as NO_3^- 0.611 g l^{-1}, P as PO_4^{3-} 0.031 g l^{-1} and K^+ at 0.236 g l^{-1}) with an extensive micronutrient supplement (Hewitt 1966). Plants from high nutrient treatments received a supplement of 300, 900, 480 and 340 mg $plant^{-1}$of P, N, K and Mg, respectively. In total, each plant (one per pot) received 1.5 l of nutrient solution. The AM inoculant was an isolate of *Glomus intraradices* (Smith & Schenck) multiplied by pot culture with maize. A non-AM inoculum was produced by growing maize in the same soil mixture after sterilization and without propagules. Maize plants were allowed to grow for 6 weeks. The roots were cut in small segments and mixed with the soil. *Columns* represent mean values (n = 10). *Columns* followed by the same *letters* within *panel* are not significantly different by Fisher's LSD test at P = 0.05

Smith (2000) described the relative growth rate of a plant (RGR) in terms of the root parameters associated with nutrient uptake:

$$\mathrm{RGR} = \mathrm{Ni} \times \mathrm{NUE} \times \mathrm{SRA} \times \mathrm{RWR}$$

Where RGR = relative growth rate, NI = net nutrient influx, NUE = nutrient use efficiency, SRA = specific root area, and RWR = root weight ratio. AM colonization can improve RGR under low nutrient conditions by a combination of morphological and physiological strategies that involve both symbionts. A large component of NI in mycorrhizal plants will be provided by the hyphae that are extremely large but almost invisible component of the SRA, since the SRA does not account for the hyphae surface area. Due to this "extra" SRA, AM plants produce relatively smaller root systems (low RWR) than the nonmycorrhizal equivalents. The organic carbon saved in the root construction is diverted to proliferation of fungus, which can access nutrients more efficiently than the equivalent plant root biomass (Smith et al. 2003). This increased efficiency of the AM root can be reflected in root architecture systems. These are modular, and each meristem can generate a potentially

unlimited number of lateral meristems (Robinson et al. 2003), resulting in exceptionally flexible architectures with a great ability to respond to environmental changes. According to Fitter et al. (1986), roots can be considered as a set of links. Links or internodes are defined as linear portions between a terminal meristem and a branching point (external link), or between two branching points (internal link). Two types of internal links can be considered: those adjacent to external links (external–internal links), and those unconnected to a link with a meristem (internal–internal links). These define where a root system is located in the continuum between the "herringbone" and dichotomous ideals.

Fitter (1991) predicted that "herringbone" root systems (i.e., those roots with a main axis and one or few developmental order of laterals) would be more expensive to construct than those with a dichotomous architecture. Dicotyledonous plants from poor habitats where acquisition of immobile soil resources is expected to be critical have more herringbone like root systems. The effect of AM colonization is to decrease the size of internodes and make the whole root system less herringbone and more dicotynomous (Cruz et al. 2004). However, this effect has only been observed under low nutrient availability, showing that the latter has a much stronger effect on root architecture than AM mycorrhiza. This is mainly due to the inhibitory effect of high nutrient availability on root colonization by AMF (Gao et al. 2001; Bouma et al. 2001). In fact, the results are in agreement with the regulatory effect of several nutrients, including nitrate, on root development and formation of lateral roots (Walch-Liu P et al. 2006).

Morphometric analysis of roots shows that the parameters affected by the AM colonization are also dependent on the plant species. For instance, *Allium porrum* (leek), *Platanus acerifolia* and *Vitis vinifera* plants colonized with *Glomus* sp. developed denser root systems, with a higher number of shorter adventitious roots of greater diameter as well as lower specific root length (SRL) than the non-AM controls (Berta et al. 1990; Schellenbaum et al. 1991; Tisserant et al. 1992) while other plant species grown under the same conditions did not.

Conclusions

AM symbiosis has a multitude of effects on plant morphology and physiology, leading in several cases to increased plant fitness. Several studies have highlighted the importance of the fungi and plant species involved in the association, as well as the need for specific abiotic conditions in order to observe plant responsiveness to AM colonization.

In this chapter, we analyzed the results obtained with two slow-growing Mediterranean species (*Ceratonia siliqua* and *Myrtus communis*) to show the importance of the AMF community on plant physiological and morphological changes in response to AM colonization and show the existence of an intrinsic relation between morphological and physiological adaptations.

The fungal communities developed after soil inoculation with *Glomus intraradices* increased shoot biomass accumulation, leaf area and enzyme activities related with

leaf nitrate reduction. They also induced higher activities of the key enzymes related with the transfer of nitrogen from the hyphae into the root (arginase and urease), indicating a direct effective contribution of the AM to nitrogen acquisition through symbiosis. However, according to the present knowledge, the increased shoot nitrate concentrations and nitrate reductase activities cannot be attributed to a direct contribution of the AMF to nitrogen acquisition. This may be explained by the effects AM symbiosis has on root physiology and morphology. It has been demonstrated that AMF increase P plant acquisition in several ways: direct acquisition through the hyphae and transference to the root, and by inducing changes in efficiency of P uptake through the plant root (Smith et al. 2004). The results presented here suggest the existence of a similar system for nitrogen, though further research with other wild and crop plants is necessary.

Figure 7 summarizes the results obtained, highlighting the fact that plant responsiveness to AM symbioses was associated with increased flow of N from the roots into the shoots and of C from the shoots into the roots. It was interesting to realise that plants responded more to the "new" AMF community induced by the inoculation of *Glomus intraradices* than to the native community. It would be interesting to study the duration of this effect and its relation with the changes in the AMF composition of the soil community (Correia 2006).

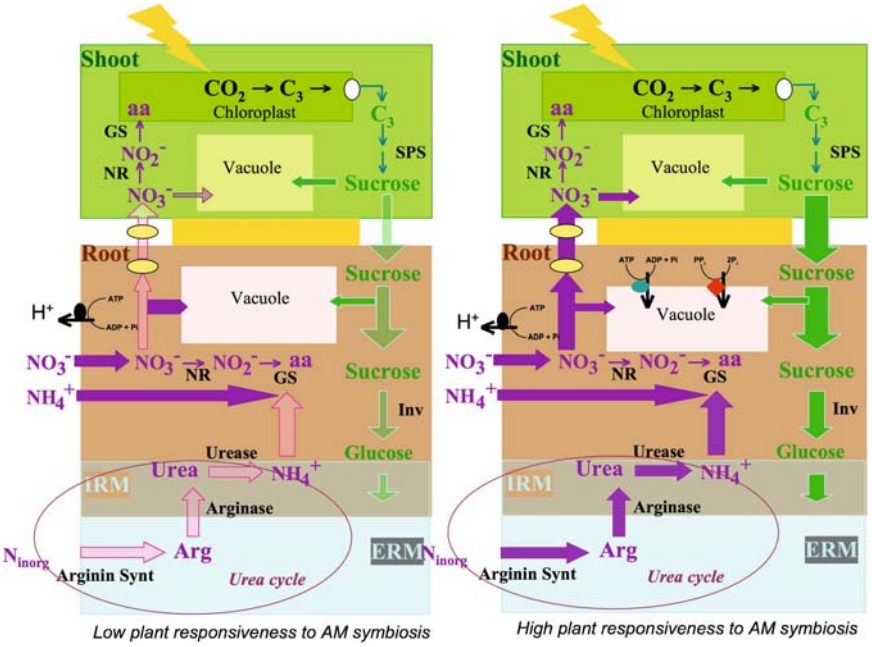

Fig. 7 Schematic representation of the effects of AM colonization related with the nitrogen acquisition by the AM hypahe and the AM root that are involved in the plant responsiveness to AM colonization. *SPS* Sucrose phosphate synthetase, *Inv* invertase, *GS* glutamine synthetase, *NR* nitrate reductase, *ERM* external root mycelium, *IRM* internal root mycelium

The results suggest that in low-resource environments, root system physiology adapts to maximize uptake capacity, and root system morphology adapts to maximize active uptake length. The role of AM is to increase the uptake capacity of the active root zone. AM colonization was higher in low-resource environments; this tendency appears to have additional growth benefits to plants from water-limited ecosystems where soil is highly heterogeneous. In these ecosystems, diffusion, and not mass flow, is the main cause of nutrient movement in the soil.

These observations demonstrate that mycorrhizal symbioses can be beneficial to plants, especially those in stressful environments, such as those of Mediterranean climates. Special attention should be given to two factors: soil management and nutrient availability. Soil management can induce changes in the composition and activity of soil AMF community, which may be determinant for plant responsiveness to AM symbiosis.

On the other hand, high levels of nutrient availability inhibit AM symbiosis. This raises the question of how increased availability of nutrients, especially nitrogen, one of the limiting factors to plant growth under Mediterranean conditions, affects mycorrhizal symbiosis.

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References

Ames RN, Reid CPP, Porter LK, Cambardella C (1983) Hyphal uptake and transport of nitrogen from two ^{15}N-labelled sources by *Glomus mossae*, a vesicular arbuscular mycorrhizal fungus. New Phytol 95:381–396

Auge RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Bago B, Pfeffer PE, Shachar-Hill Y (2000) Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol 12:949–957

Bago B, Pfeffer P, Shachar-Hill Y (2001) Could the urea cycle be translocating nitrogen in the arbuscular mycorrhizal symbiosis? New Phytol 149:4–8

Bago B, Vierheilig H, Piche Y, Azcón Aguilar C (1996) Nitrate depletion and pH changes induced by the extra-radical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytol 133:273–280

Baylis GTS (1975) The magnoid mycorrhiza and mycotrophy in root system derived from it. In: Sanders EF, Moose B, Turker PB (eds) Endomycorrhizas. Academic, New York, pp 373–389

Berta G, Fusconi A, Trotta A, Scannerini S (1990) Morphogenetic modifications induced by the mycorrhizal fungus *Glomus* strain E3 in the root system of *Allium porrum* L. New Phytol 114:207–215

Berta G, Fusconi A, Trotta A (1993) VA mycorrhizal infection and the morphology and function of root systems. Environ Exp Bot 33:159–173

Black KG, Mitchell DT, Osborne BA (2000) Effect of mycorrhizal-enhanced leaf phosphate status on carbon partitioning, translocation and photosynthesis in cucumber. Plant Cell Environ 23:797–809

Blee KA, Anderson AJ (1998) Regulation of arbuscule formation by carbon in the plant. Plant J 16:523–530

Bouma TJ, Yanai RD, Elkin AD, Hartmond U, Flores-Alva DE, Eissenstat DM (200) Estimating age-dependent costs and benefits of roots with contrasting life span: comparing apples and oranges. New Phytol 150:685–695

Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159:567–584

Caravaca F, Al Guacil MM, Hernandez JA, Roldan A (2005). Involvement of antioxidant enzyme and nitrate reductase activities during water stress and recovery of mycorrhizal *Myrtus communis* and *Phillyrea angustifolia* plants. Plant Sci 169:191–197

Chiou TJ, Aung K, Lin S-I, Wu CC, Chiang S-F, Sua C-L (2005) Regulation of phosphate homeostasis by microrna in *Arabidopsis*. Plant Cell 18:412–421

Cliquet J-B, Stewart GR (1993) Ammonia assimilation in *Zea mays* L. infected with a vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum*. Plant Physiol 101:865–871

Comas LH, Eissenstat DM, Lakso AN (2000) Assessing root death and root system dynamics in a study of grape canopy pruning. New Phytol 147:171–178

Correia P (2006) The role of mycorrhizae in Mediterranean ecosystem revegetation. PhD thesis, Universidade de Lisboa

Cruz C, Green JJ, Watson CA, Wilson F & Martins-Loução MA (2004) Functional aspects of root architecture and mycorrhizal inoculation with respect to nutrient uptake capacity. Mycorrhiza 14:177–184

Cruz C, Egsgaard H, Trujillo C, Ambus P, Requena N, Martins-Loução MA, Jakobsen I (2007) Enzymatic evidence for the key role of arginine in nitrogen translocation by arbuscular mycorrhiza fungi. Plant Physiol. 144:782–792

Derner JD, Briske DD (1999) Does a tradeoff exist between morphological and physiological root plasticity? Comparison of grass growth forms. Acta Oecol 20:519–526

Douds DD, Pfeffer PE, Shachar-Hill Y (2000) Carbon partitioning, cost and metabolism of arbuscular mycorrhizae in arbuscular mycorrhizas: physiology and function. In: Kapulnick Y, Douds DD Jr (eds) Arbuscular mycorrhizas: molecular biology and physiology. Kluwer, Dordrecht, pp 107–129

Eckstein RL, Karlsson PS (2001) Variation in nitrogen-use efficiency among and within subarctic graminoids and herbs. New Phytol 150:641–651

Ferrol N, Gianinazzi S, Gianinazzi-Person V (2002) Arbuscular mycorrhiza induced ATPases and membrane nutrient transport mechanisms. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture: from genes to bioproducts. Birkhäuser, Basel, pp 113–122

Fitter AH (1986) The topology and geometry of plant root systems: influence of watering rate on root system topology in *Trifolium pratense*. Ann Bot 58:91–101

Fitter AH (1991) Characteristics and functions of root systems. In: Waisel Y, Eshel A, Kafkafi Y (eds) Plant roots: the hidden half. Dekker, New York, pp 3–25

Fitter AH (1991) The ecological significance of root system architecture: an economic approach. In: Atkinson D (ed) Plant root growth: an ecological perspective. Blackwell, Oxford, pp 229–243

Fitze D, Wiepning A, Kaldorf M, Ludwig-Muller J (2005) Auxins in the development of an arbuscular mycorrhizal symbiosis in maize. J Plant Physiol 162:1210–1219

Flores E, Frías JE, Rubio LM, Herrero A (2005) Photosynthetic nitrate assimilation in cyanobacteria. Photosyn Res 83:117–133

Gao L-L, Delp G, Smith SE (2001) Colonization patterns in a mycorrhiza-defective mutant tomato vary with different arbuscular-mycorrhizal fungi. New Phytol 151:477–491

George E, Marschner H, Jakobsen I (1995) Role of arbuscular mycorrhizal fungi in uptake of phosphorus and nitrogen from soil. Crit Rev Biotech 15:257–270

Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith FA (1991) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas. V. is H^+-ATPase a component of ATP-hydrolysing enzyme activities in plant-fungus interfaces? New Phytol 117:61–57

Gianinazzi-Pearson V, Arnould C, Oufattole M, Arango M, Gianinazzi (2000) Differential activation of H^+-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. Planta 211:609–613

Gonçalves MT (2000) Micorrizas em *Populus nigra* L. Aspectos citológicos e fisiológicos. PhD thesis, Universidade de Coimbra

Gonçalves MT, Martins-Loução MA (1997) The effect of two arbuscular mycorrhiza fungi on growth and phosphorus nutrition of *Populus nigra* seedlings. In: Ando T, Fujita K, Mae T, Matsumoto H, Mori S, Sekiya J (eds) Plant nutrition for sustainable food production and environment. Kluwer, Dordrecht, pp 727–732

Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–823

Graham JH, Abbott LK (2000) Wheat responses to aggressive and non-aggressive arbuscular mycorrhizal fungi. Plant Soil 220:207–218

Graham JH, Duncan LW, Eissenstat, DM (1997) Carbohydrate allocation patterns in citrus genotypes as effected by phosphorus nutrition, mycorrhizal colonisation and mycorrhizal dependency. New Phytol 135:335–343

Handley LL, Azcon R, Ruiz Lozano JM, Scrimgeour CM (1999) Plant ^{15}N associated with arbuscular mycorrhiza, drought and nitrogen deficiency. Rapid Commun Mass Spectrom 13:1320–1324

Harris D, Paul EA (1987) Carbon requirements of vesicular-arbuscular mycorrhiza. In: Ecophysiology of VA mycorrhizal plants. CRC, Boca Raton, Fla., pp 93–105

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Hawkins HJ, Johansen A, George E (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. Plant Soil 226:275–285

Hewitt EJ (1966) Sand and water culture methods used in the study of plant nutrition. Technical communication No. 22. Commonwealth Agricultural Bureaux. Eastern Press, London

Hobbie EA, Macko SA, Williams M (2000) Correlations between foliar 15 N and nitrogen concentrations may indicate plant–mycorrhizal interactions. Oecologia 122:273–283

Hooker JE, Black KE, Perry RL, Atkinson D (1995) Arbuscular mycorrhizal fungi induced alteration to root longevity of poplar. Plant and Soil 172:327–329

Jakobsen I, Smith SE, Smith FA (2002) Function and diversity of arbuscular mycorrhizae in carbon and mineral nutrition. In: van der Heijden M, Sanders IR (eds) Mycorrhizal ecology. Springer, Heidelberg, pp 85–92

Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y (2005) The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol 168:687–696

Johansen A, Jakobsen I, Jensen ES (1992) Hyphal transport of ^{15}N-labelled nitrogen by a vesicular-arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil N. New Phytol 122:281–288

Jones FR (1924) A mycorrhizal fungus in the roots of legumes and some other plants. J Agri Res 24:459–470

Kaldorf M, Schmelzer E, Bothe H (1998) Expression of maize and fungal nitrate reductase genes in arbuscular mycorrhiza. Mol Plant Microbe Interact 11:439–448

Lambers H, Chapin FS III, Pons TL (1998) Plant physiological ecology. Springer, Heidelberg

Li X L, Marschner H and George E (1991) Acquisition of phosphorus and copper by VA-mycorrhizal hyphae and root–shoot transport in white clover. Plant Soil 136:49–57

Liu J, Blaylock LA, Endre G, Cho J, Town CD, VandenBosch KA Harrison MJ (2003) Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. Plant Cell 15:2106–2123

Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T (2005) Organization and metabolism of plastids and mitochondria in arbuscular mycorrhizal roots of *Medicago truncatula*. Plant Physiol 139:329–340

López B, Sabaté S, Gracia CA (2001) Fine-root longevity of *Quercus ilex*. New Phytol 151:437–441

Majdi H, Damm E, Nylund J-E (2001) Longevity of mycorrhizal roots depends on branching order and nutrient availability. New Phytol 150:195–202

Martins-Loução MA, Cruz C (1999) The role of N source on carbon balance. In: Srivastava HS, Singh RP (eds) Modes of nitrogen nutrition in higher plants. Oxford and IBM, New Delhi, pp 231–282

Martins-Loução MA, Lips SH (2000) Nitrogen in a sustainable ecosystem: a matter of integration. In: Martins-Loução MA, Lips SH (eds) Nitrogen in a sustainable ecosystem: from the cell to the plant. Backhuys, Leiden, pp 415–419

Munsell (1975) Soil colour chart. Munsell Colour, Baltimore

Paszkowski U (2006) A journey through signaling in arbuscular mycorrhizal symbioses 2006. New Phytol 172:35–46

Pirozynski KA, Dalpé Y (1989) The geological history of the *Glomaceae* with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–36

Poorter L (2001) Light-dependent changes in biomass allocation and their importance for growth of rain forest tree species. Funct Ecol 15:113–123

Remy W, Taylor TN, Hass H and Kerp H (1994) Four hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Robinson D, Hodge A, Fitter A (2003) Constrains on the form and function of root systems. In: de Kroon H, Visser EJW (eds). Root ecology. Ecological studies 168. Springer, Heidelberg, pp 1 - 28

Ruiz-Lozano JM (2003) Arbiscular mycorrhiza symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13:309–317

Schaarschmidt S, Roitsch T, Hause, B (2006) Arbuscular mycorrhiza induces gene expression of the apoplastic invertase LIN6 in tomato (*Lycopersicon esculentum*) roots. J Exp Bot 57:4015–4023

Schellenbaum L, Berta G, Ravolanirina F, Tisserant B, Gianinazzi S, Fitter AH (1991) Influence of endomycorrhiza infection on root morphology in a micropropagated woody plant species *(Vitis vinifera L.)*. Ann Bot 68:135–141

Schliemann W, Schmidt J, Nimtz M, Wray V, Fester T, Strack D (2006) Apocarotenoids from mycorrhizal roots of *Ornithogalum umbellatum*. Phytochemistry 67:1196–1205

Smith FA (2000). Measuring the influence of mycorrhizas. New Phytol 148:4–6

Smith FA, Smith SE, Timonen E (2003) Mycorrhizas. In de Kroon H, Visser EJW (eds). Root ecology. Ecological studies 168. Springer, Heidelberg. pp 257- 296.

Smith SE, Read, DJ. 1997. Mycorrhizal symbiosis, 2nd edn. Academic, London

Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol 133:16–20

Smith SE, Smith FA, Jakobsen I (2004) Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol 162:511–524

Spriggs AC, Stock WD, Dakora FD (2003) Influence of mycorrhizal associations on foliar 15N values of legume and non-legume shrubs and trees in the fynbos of South Africa: implications for estimating N_2 fixation using the ^{15}N natural abundance method. Plant Soil 255:495–502

Strack D, Fester T (2006) Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. New Phytol 172:22–34

Taylor J, Harrier LA (2003) Expression studies of plant genes differentially expressed in leaf and root tissues of tomato colonised by the arbuscular mycorrhizal fungus *Glomus mosseae*. Plant Mol Biol 51:619–629

Tisserant B, Schellenbaum L, Gianinazzi V, Gianinazzi S, Berta G (1992) Influence of infection by an endomycorrhizal fungus on root development and architecture in *Platanus acerifolia*. Allionia 30:171–181

Toussaint J-P (2007) Investigating physiological changes in the aerial parts of AM plants: what do we know and where should we be heading? Mycorrhiza 17:349–353

Walch-Liu P, Liu L-H, Remans T, Tester M, Forde BG (2006) Evidence that L-Glutamate can act as an exogenous signal to modulate root growth and branching in *Arabidopsis thaliana*, Plant Cell 47:1045–1057

Wright SF, Upadhyaya A (1998) A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant Soil 198:97–107

Wright SF, Upadhyaya A, Buyer JS (1998) Comparison of N-linked oligosaccharides of glomalin from arbuscular mycorrhizal fungi and soils by capillary electrophoresis. Soil Biol Biochem 13:1853–1857

Novel Symbiotrophic Endophytes

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1 Introduction

Endophytes are microorganisms that reside in the tissues of living plants without causing any immediate overt negative effect (Bacon and White 2000). Of the nearly 300,000 plants species that exist on the earth, each individual plant is host to one or more endophytes (Strobel and Daisy 2003). These endophytes are relatively unstudied and are potential sources of novel natural products for exploitation in medicine, agriculture, and industry. Very few of these endophytes have been studied to date, and this opens a new opportunity to find out novel endophytes in myriads of ecological niches. One of the important facets of these endophytes is their evolution with plants over a period of millennia that makes them live in a symbiotrophic relationship in such a way that both becomes indispensible to each other. While the symptomless nature of endophyte occupation in plant tissue has prompted the focusing on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens (Strobel and Daisy 2003).

The most common endophytes of plants are bacteria and fungi, but evidence indicates that other life forms like viruses exist in plants as endophytes (Marquez et al. 2007). The most frequently isolated endophytes are the fungi (Hawksworth and Rossman 1987). Dreyfuss and Chapela (1994) estimated that there may be at least 1 million species of endophytic fungi alone. It can be readil concluded that majority of these endophytes are symbiotic and can have tremendous applications in the area of drug discovery, medicine and agriculture.

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2 Strategy for Endophyte Discovery

Several reasonable hypotheses (Strobel and Daisy 2003) govern the plant selection strategy for selection of novel symbiotrophic endophytes and these are:

1. Plants from extreme environmental settings and with unusual biology possessing novel strategies for survival.
2. Plants having ethnobotanical history. The healing source may reside in the endophyte.
3. Plants which have unusual longevity are more likely to be lodged with endophytes possessing natural products.
4. Plants living in the areas with high biodiversity also have the prospect of housing novel endophytes

2.1 Extreme Environments and Endophytes Discovery

Extreme environmental conditions and unusual plant biology offers great opportunities for the discovery of novel symbiotrophic endophytes (Rodriguez et al. 2004a). For example, *Piriformospora indica*, a novel multifunctional symbiotrophic fungal endophyte (Verma et al. 1998) was discovered from the Great Indian Desert of Western Rajasthan in India. The fungus was found to colonize roots of the desert plants which were growing in extreme conditions of water scarcity. Subsequently, it was established that the fungus promotes acquisition of drought tolerance in plants (Waller et al. 2005; Sherameti et al. 2008b).

Rhyncholacis penicillata, an aquatic plant from southwest Venezuela is subjected to constant pummeling by virtue of rushing waters, debris, and tumbling rocks and pebbles (Strobel et al. 1999). This created many portals through which common phyopathogenic fungi could enter the plant. Still, the plant population appeared to be healthy, possibly due to presence of an endophytic product. This was the environmental clue to examine the plant for the presence of novel symbiotrophic endophytes. Eventually, a potent antifungal strain of *Serratia marcescens* was recovered from *R. penicillata* and was shown to produce oocydin A, a novel antioomycetous compound having the properties of a chlorinated macrocyclic lactone. The production of oocydin A by *S. marcescens* helped the plant to ward off the invasion of phtyopathogenic oomycetes. Currently, oocydin A is being considered for agricultural use to control the ever-threatening presence of oomyceteous fungi such as *Pythium* and *Phytophthora*.

A plant–fungal symbiosis between a tropical grass from geothermal soils, *Dichanthelium lanuginosum*, and the fungus *Curvularia protuberata* allows both organisms to grow at high soil temperatures in Yellowstone National Park USA (Redman et al. 2002). Field and laboratory experiments have shown that when root zones are heated up to 65 °C, nonsymbiotic plants either become shriveled and chlorotic or simply die, whereas symbiotic plants tolerate and survive the heat

regime. When grown separately, neither the fungus nor the plant alone is able to grow at temperatures above 38 °C, but symbiotically they are able to tolerate elevated temperatures. In the absence of heat stress, symbiotic plants have enhanced growth rates compared with nonsymbiotic plants and also show significant drought tolerance (Rodriguez et al. 2004b). Recently, the presence of a virus having dsRNA was detected in the fungus *C. protuberata* by Marquez et al. (2007). The virus particles purified from *C. protuberata* were similar to those of other fungal viruses and ~27 nm in diameter. The virus was named as *Curvularia* thermal tolerance virus (CThTV) to reflect its host origin and phenotype. The ability of the fungus to confer heat tolerance to its host plant is related to the presence of CThTV. Wild type isolates of *C. protuberata* contained the virus in high titers as evidenced by their high concentration of ds RNA (~2 µg/g of lyophilized mycelium). However, an isolate obtained from sectoring (change in morphology) of a wild type colony contained a very low titre of virus, as indicated by low concentration of dsRNA (~0.02 µg/g of lyophilized mycelium). The mycelium of the virus obtained by sectoring was made completely virus free by lyophilization, freezing at −80 °C and further subculturing. Subsequently, the ability of the wild type and virus-free isolates to confer heat tolerance by using thermal soil stimulators was assessed experimentally (Redman et al. 2002). Plants inoculated with the virus-infected wild type isolate of the fungus tolerated intermittent soil temperatures as high as 65 °C for 2 weeks (10 h of heat per day), whereas both nonsymbiotic plants and plants inoculated with the virus-free isolate of the fungus became shriveled and chlorotic and died. To confirm that CThTV was involved in heat tolerance in the plant–fungal symbiosis, the virus was reintroduced into the virus-free fungal isolate and its ability to confer heat tolerance was tested. To provide a selectable marker, the virus-free isolate was transformed with a pCT74 vector containing a hygromycin-resistance gene (Lorang 2001) by restriction enzyme-mediated integration (REMI) transformation (Redman et al. 1999). Virus-containing wild type hygromycin-sensitive (Wt) and virus-free hygromycin-resistant (VF) isolates of *C. protuberata* were cultured on single Petri dishes and allowed to undergo hyphal fusion or anastomosis. The mycelium from the area of anastomosis was subcultured twice with single conidiospores grown on hygromycin containing plates. Thirty-five hygromycin-resistant isolates obtained in this way were screened for their dsRNA profiles, but only one was found to have acquired the virus. This fungal isolate, newly infected by hyphal anastomosis with CThTV (An), was tested for its ability to confer heat tolerance by the same experimental approach indicated above. The heat-stress experiment confirmed that the isolate newly infected with CThTV confers the same level of heat tolerance as that conferred by the wild type isolate. Furthermore, the ability of the *C. protuberata* isolates to confer heat tolerance to tomato (*Solanum lycopersicon*) was also tested. Using a slightly modified protocol for the heat-stress experiment similar results were obtained as with *D. lanuginosum*. However, it was not possible to attain 100% fungal colonization of tomato plants, and this may explain the higher proportion of dead plants colonized with the Wt or An fungus, compared with the experiment using *D. lanuginosum*. Given that *C. protuberata*, when infected with CThTV, provides similar mutualistic benefits to both a monocot- and eudicot, it is possible that

the underlying mechanism is conserved between these two groups of plants. Plants inoculated with *C. protuberata* infected with CThTV do not activate their stress-response system in the usual way. For example, the osmolyte concentration in these plants does not increase as a response to heat stress, although the levels were constitutively higher than in plants colonized with the virus-free isolate or the non-symbiotic plants. It has been hypothesized that endophytes may protect their host plants by scavenging the damaging reactive oxygen species (ROS) generated by the plant defense mechanisms in response to environmental stress (Rodriguez and Redman 2001). The leaves of nonsymbiotic plants generated detectable ROS when stressed with heat, whereas those of symbiotically colonized plants did not. However, there was no difference in the ROS response to heat between plants inoculated with the virus-free and the CThTV-infected isolates of *C. protuberata*.

This type of complex tripartite symbioses have been found among arthropods, bacteria, and mutualistic bacteriophages (Bordenstein and Wernergreen 2004; Moran et al. 2005). This study reports a three-way mutualistic symbiosis involving a virus, a fungal endophyte, and either a monocot or eudicot plant.

2.2 Plants with Ethnobotanical History

Plants with ethnobotanical history are likely candidates to harbor novel symbiotrophic endophytes. The beneficial medical use of the selected plant may reside in its symbiotic endophyte rather than in the plant's phtyochemistry. For example, *Kennedia nigriscans* from the Northern Territory of Australia has been traditionally used as bush medicine for many years by one of the world's long standing civilizations – the Australian Aborigines – for treatment of cuts, wounds, and infections. When analyzed, it was found that the plant contained a novel symbiotrophic endophyte, *Streptomyces* sp. Strain NRRL 30562, that produces wide-spectrum novel peptide antibiotics called munumbicins (Castillo et al. 2002).

2.3 Plants Having Unusual Longevity

Production of certain bioactive compounds by the endophyte in situ may facilitate the domination of its biological niche within the plant or even provide protection to the plants from harmful invading pathogens. For example, *Pestalotiopsis jesteri* a novel endophyte described from the rainforests of Papua Guinea produces jesterone and hydroxyl-jesterone, which exhibit antifungal activity against a variety of plant-pathogenic fungi (Li and Strobel 2001). Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp. (from the leaves of *Aspidosperma tomentosum* and twigs of *Spondias mombin* and other plants), represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite mainly exhibits antibacterial activity against *Bacillus subtilis, Salmonella enterica*

and *Staphylococcus aureus*. It also displays moderate activity against the yeast *Candida tropicalis* (Horn et al. 1995).

Colletotric acid, a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*, displays antimicrobial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou et al. 2000). Another *Colletotrichum* sp., isolated from *Artemisia annua*, produces bioactive metabolites that showed varied antimicrobial activity as well. The *Colletotrichum* sp. found in *A. annua* produced not only metabolites with activity against human-pathogenic fungi and bacteria but also metabolites that were fungistatic to plant-pathogenic fungi (Lu et al. 2000).

Another endophytic streptomycete (NRRL 30566), from a fern-leaved *Grevillea* tree (*Grevillea pteridifolia*) growing in the Northern Territory of Australia, produces, in culture, novel antibiotics called kakadumycins (Castillo et al. 2003). Each of these antibiotics contain, by virtue of their amino acid compositions, alanine, serine, and an unknown amino acid. Kakadumycin A which has wide-spectrum antibiotic activity especially against gram-positive bacteria.

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong et al. 2001). This fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds (Strobel et al. 2001). The majority of these compounds have been identified by gas chromatography-mass spectrometry, synthesized or acquired, and then ultimately made into an artificial mixture. This mixture mimicked the antibiotic effects of the volatile compounds produced by the fungus. It was also used to gain positive identification of the ingredients of the fungal volatile compounds (Strobel et al. 2001). Each of the five classes of volatile compounds produced by the fungus had some inhibitory effect against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant- and human-pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active.

Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2-(1′-hydroxy-5′-methyl-4′-hexenyl) benzofuran and 5-hydroxy-2-(1′-oxo-5′-methyl-4′-hexenyl) benzofuran, both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm (Findlay et al. 1997). Another endophytic fungus, *Muscodor vitigenus*, isolated from a liana (*Paullina paullinioides*), yields naphthalene as its major product. Naphthalene, the active ingredient in common mothballs, is a widely exploited insect repellant. *M. vitigenus* shows promising preliminary results as an insect deterrent and has exhibited potent insect repellency against the wheat stem sawfly (*Cephus cinctus*) (Daisy et al. 2002a, 2002b).

2.4 Plants Growing in Biodiversity Hotspots

Among the different ecosystems on earth, those having the greatest biodiversity seem to be the ones also having endophytes with the greatest number and the most biodiverse microorganisms. Tropical and temperate rainforests are the most

biologically diverse terrestrial ecosystems on earth. The most threatened of these spots cover only 1.44% of the land's surface, yet they harbor more than 60% of the world's terrestrial biodiversity (Mittermeier et al. 1999). As such, one would expect that areas of high plant endemicity also possess specific endophytes that may have evolved with the endemic plant species.

Tropical rainforests are a remarkable example of this type of environment and it is highly probable that rainforests are a source of novel molecular structures and biologically active compounds (Redell and Gordon 2000). For example a nonpeptidal fungal metabolite (L-783,281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang et al. 1999). This compound acts as insulin mimetic and, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783,281 to two mouse models of diabetes resulted in significant lowering of blood glucose levels.

3 Case Study: *Priformospora indica* – A New Champion of Symbiosis

Scientists from the Amity University Uttar Pradesh, Noida, have discovered a novel endophytic fungus, *Piriformospora indica*, which mimics the capabilities of typical, AM fungus. *P. indica* is a recently isolated root-interacting fungus, related to the Hymenomycetes of the Basidiomycota (Verma et al. 1998). In contrast with arbuscular mycorrhizal fungi, it can be easily cultivated in axenic culture where it produces chlamydospores (Oelmüller et al. 2005; Peškan-Berghöfer et al. 2004; Shahollari et al. 2005). The fungus is able to associate with the roots of various plant species in a manner similar to mycorrhiza and promotes plant growth (Varma et al. 1999, 2001; Singh An et al. 2002, 2003a; Oelmüller et al. 2004; Peškan-Berghöfer et al. 2004; Pham et al. 2004a; Shahollari et al. 2005). Pronounced growth promotional effects were also seen with terrestrial orchids (Blechert et al. 1999). The fungus can easily be cultivated on a number of synthetic and complex media (Hill and Käefer 2001; Pham et al. 2004b).

P. indica tremendously improves the growth and overall biomass production of diverse hosts, including legumes, medicinal and economically important plants (Varma et al. 1999; Varma et al. 2000). The plants tested in the laboratory conditions as well as in the extensive field trial were *Bacopa monieri, Nicotiana tobaccum* (Sahay and Varma 1999, 2000), *Artemisia annua, Petroselinum crispum* (Varma et al. 1999), *Azadirachta indica* (Singh An et al. 2002; Singh et al. 2003a), *Tridex procumbans, Abrus precatorius* (Kumari et al. 2004), *Chlorophytum borivilianum* (Pham et al. 2004a), *Withania somnifera* and *Spilanthes calva* (Rai et al. 2001) and *Adhatoda vasica* (Rai and Varma 2005). *P. indica* promotes antifungal potential of medicinal plant *Spilanthus calva* due to increase in spilanthol content after interaction (Rai et al. 2004). Experiments on maize (Varma et al. 1999) and cabbage (Kumari et al. 2003) have demonstrated the enhanced biomass production as a result of fertilization with the fungus culture filtrate. The fungus also has the

potential to act as bioprotectant against fungus root pathogens and soil insects (Pham et al. 2004c). *P. indica* promises to be an excellent agent for biological hardening of tissue culture-raised plants, as the fungus rendered more than 90% survival rate of the transferred plantlets of these plants and, by excessive root proliferation and induction of secondary rootlets, protects them from 'transplantation shock' and potent root pathogens (Singh Ar et al. 2002; Singh et al. 2003b; Varma et al. 2000). Therefore, this fungus promises to be a boon for the plant industries (Hazarika 2003; Singh et al. 2003a).

Among the compounds released in root exudates, flavonoids are found to be present in *P. indica*. Flavonoids have been suggested to be involved in stimulation of precontact hyphal growth and branching (Gianinazzi-Pearson et al. 1989; Siqueira et al. 1991), which is consistent with their role as signaling molecules in other plant-microbe interactions (Giovannetti and Sbrana 1998). Cell wall degrading enzymes like CMCase, polygalactouronase and xylanase were found in significant quantities both in the culture filtrate and in the roots colonized with *P. indica*.

P. indica showed profound effects on disease control when challenged with a virulent root and seed pathogen *Gaeumannomyces graminis. P. indica* completely blocked growth of this pathogen. It indicates that *P. indica* acted as a potential agent for biological control of root diseases, although the chemical nature of the inhibitory factor is still unknown (Varma et al. 2001). *P. indica* has shown profound effects on controlling the disease as and when challenged with a virulent root and seed pathogens. An interaction study was conducted with soil pathogenic fungi. It was observed that *P. indica* was inhibitory to the growth of these pathogenic fungi. A demarcating colour line was recorded at the contact zone of symbiotic fungi with pathogenic fungi (Fig. 1). The growth inhibition of the pathogens may be due to some enzyme

Fig. 1 Interaction of *P. indica* with *Alternaria solani*

or some diffusible toxins produced by *P indica*. The restricted growth of the pathogen directly indicates the growth promoting and protective properties of the fungus *P. indica* against root pathogens and insects to the economically important plants.

On interaction of *P. indica* with different strains of plant growth promoting rhizobacteria (PGPRs), it was observed that the growth was completely blocked by *Pseudomonas fluorescence*, however, *Azotobacter chroococcum* promoted the growth of the fungus (Pham et al. 2004a).

Interaction of *P. indica* with *Arabidopsis* roots mimics an arbuscular mycorrhiza. A MATH domain-containing (MATH) protein at the plasma membrane of *Arabidopsis* roots is one of the first components which respond to the presence of this fungus (Oelmüller et al. 2005). *P. indica* promotes growth of *Arabidopsis* and tobacco seedlings and stimulates nitrogen accumulation and the expression of the genes for nitrate reductase and the starch-degrading enzyme glucan-water dikinase (SEX1) in roots (Sherameti et al. 2005). It also stimulates the expression of the uidA gene under the control of the *Arabidopsis* nitrate reductase (Nia2) promoter in transgenic tobacco seedlings. At least two regions (_650/_628 and _103/_89) are important for Nia2 promoter activity in tobacco roots. One of the regions contains an element, TTCTAGAGT that binds to a homeo-domain transcription factor in vitro. The message for this transcription factor is up regulated by *P. indica*. The transcription factor also binds to a TACTAGATT segment in the SEX1 promoter in vitro.

Eighteen-day-old *Arabidopsis* seedlings, which were either cocultivated with the fungus or mock-treated for the last 9 days, were exposed to mild drought stress for 84 h. During the first 36–48 h, seedlings cocultivated with the fungus continued to grow, while the noncolonized controls did not (Sherameti et al. 2008b). This resulted in a 3-fold difference in the fresh weight and >2-fold difference in the chlorophyll content. The photosynthetic efficiency was only slightly reduced in the colonized (Fv/Fm at t0h = 0.82 and t36h = 0.79) and severely impaired in the noncolonized seedlings (Fv/Fm at t0h = 0.81 and t36h = 0.49), which also showed symptoms of withering. When seedlings exposed to drought stress for 72 or 84 h were transferred to soil, 10% (72 h) and none (84 h) of the noncolonized seedlings reached the flowering stage, while 59% (72 h) and 47% (84 h) of the colonized seedlings flowered and produced seeds. After 3 h exposure to drought stress, the message levels for response to dehydration 29A, early response to dehydration1, ANAC072, dehydration-response element binding protein2A, salt- and drought-induced ring finger1, phospholipase Dd, calcineurin B-like protein (CBL)1, CBL-interacting protein kinase3 and the histone acetyltransferase were upregulated in the leaves of *P. indica*-colonized seedlings. Noncolonized seedlings responded 3–6 h later and the message levels increased much less. An *Arabidopsis* ethylmethane-sulfonate mutant was identified, which was less resistant to drought stress and in which the stress-related genes were not upregulated in the presence of *P. indica*. Thus, *P. indica* confers drought stress tolerance to *Arabidopsis*, and this is associated with the priming of the expression of a quite diverse set of stress-related genes in the leaves. Transfer to soil is again associated with a faster and stronger upregulation of the message levels for phospholipase Dd, CBL1 and histone acetyltransferase in

P. indica-colonized seedlings, indicating that this response might also contribute to better survival on soil.

Arabidopsis plants colonized with the fungus produce 22% more seeds than noncolonized plants (Shahollari et al. 2007). It was found that pii-2, and presumably also At5g16590, two proteins present in plasma membrane microdomains, appear to be involved in *P. indica*-induced growth promotion and enhanced seed production in *Arabidopsis thaliana*. However, the ethyl methane-sulfonate mutant *P. indica-insensitive 4 (pii-4)* (and a corresponding T-DNA insertion line) shows little root colonization and the seedlings did not responded to the fungus (Sherameti et al. 2008a). During later stages of development, some growth promotion can be observed, although the overall response to *P. indica* was reduced compared to the wild-type. The mutants produced 2–7% more seeds in the presence of the fungus, while wild type plants produce >20% more seeds. The *NAI1* (At2g22770) transcript level was at the detection limit in *pii-4*, presumably because of an 8-bp deletion directly upstream of the *NAI1* ATG codon. NAI1 is a transcriptional activator of *PYK10*, a gene for a â-glucosidase located in endoplasmatic reticulum (ER)-derived organellar structures called ER bodies (Matsushima et al. 2004). Both *PYK10* transcript and PYK10 protein levels were severely reduced in *pii-4* and in the T-DNA insertion line. Seedlings with a T-DNA insertion in PYK10 also fail to respond to *P. indica* indicating that PYK10 and not NAI1 was responsible for the proper response to the fungus. Thus, either PYK10 itself or the ER bodies were involved in early steps of the interaction between the two symbiotic partners and the promotion of seed production in adult *Arabidopsis* plants.

After interaction with *P. indica, Nicotiana attenuata* plants realize a fitness benefit and it was suggested that the enhanced fitness comes at the expense of a reduction in resistance against herbivores (Barazani et al. 2005).

The fungus serves as a good model organism to study phosphorus metabolism (Malla et al. 2005). The fungus grows on a large varieties of inorganic, organic and polyphosphates. The molecular mass of denatured acid phosphatase (ACPase) of *P. indica* was found to be 66 kD in SDS PAGE. *P. indica* has been reported to induce resistance in the monocotyledonous plant barley to fungal diseases, along with tolerance to salt stress without affecting the plant productivity (Waller et al. 2005). The beneficial effect on the defense status is detected in distal leaves demonstrating a systemic induction of resistance. The systemically altered "defence readiness" is associated with an elevated antioxidative capacity due to an activation of the glutathione-ascorbate cycle and an overall increase in grain yield. The fungus has been analyzed for its symbiotic interaction and endophytic development in barley (Deshmukh et al. 2006). It was found that the fungal colonization increases with root maturation. The root tip meristem showed no colonization, and the elongation zone showed mainly intercellular colonization. In contrast the differentiation zone was heavily infested by inter- and intracellular Hyphae and intracellular chlamydospores. In some cases, hyphae penetrated cells and built a meshwork around plasmolyzed protoplasts, suggesting that the fungus either actively kills cells or senses cells undergoing programmed cell death. Seven days after inoculation, expression of barley BAx inhibitor-1 (HvBl-1), a gene capable of initiating plant

cell death was attenuated. Consistently, fungal proliferation was strongly inhibited in transgenic barley over expressing GFP-tagged HvBl-1, which shows that *P. indica* requires host cell death for proliferation in differentiated barley roots. It was suggested, therefore, that the fungus interferes with the host cell death program to form a mutualistic interaction with plants. Interaction with *Populus* Esch5 revealed that *P. indica* could be directed in its physiological behavior from mutualistic to antagonistic by specifically designed cultural conditions (Kaldorf et al. 2005) hence making it a potential model system to study plant–microbe interactions. It provides a promising model organism for the investigation of beneficial plant–microbe interactions, and enables the identification of compounds, which may improve plant growth, productivity and maintain soil productivity. The various multifunctional roles of *P. indica* are outlined below (Fig. 2).

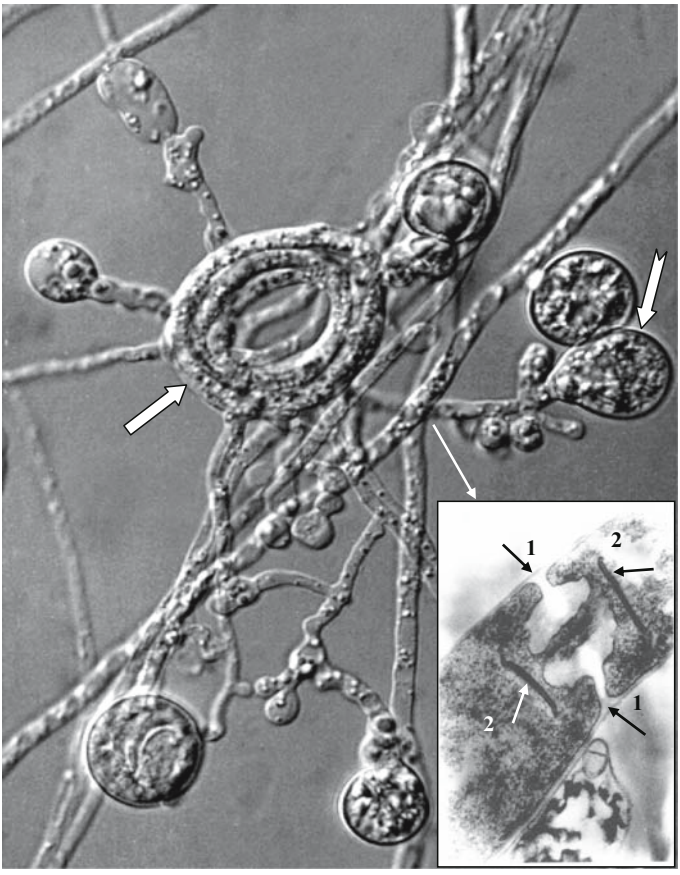

Fig. 2 Multifunctional symbiotic fungus *Piriformospora indica*

4 Conclusions

Symbiotrophic endophytes are a relatively poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial uses. The mechanisms through which endophytes exist and respond to their surroundings must be better understood in order to be more predictive about the discovery of novel symbiotrophic endophytes. One of the serious hurdles in discovering novel endophytes is the unprecedented loss of biodiversity which is taking place, epecially the way tropical rainforest are depleting, which are thought to possess more of these novel microbes than any other ecological system. The multifunctionality of the new champion of symbiosis, i.e., *P. indica* once again emphasizes the fact that equal importance should be given to each and every living creature that exists on this planet.

References

Bacon CW, White JF (2000) Microbial endophytes. Dekker, New York

Barazani O, Benderoth M, Groten K, Kuhlemeier C, Baldwin IT (2005) *Piriformospora indica* and *Sebacina vermifera* increase growth performance at the expense of herbivore resistance in *Nicotiana attenuata*. Oecologia 146:234–243

Blechert O, Kost G, Hassel A, Rexer R-H, Varma A (1999) First remarks on the symbiotic interactions between *Piriformospora indica* and terrestrial orchids. In: Varma A, Hock B (eds) Mycorrhizae, 2nd edn, Springer, Heidelberg, pp 683–688

Bordenstein SR, Wernegreen JJ (2004) Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. Mol Biol Evol 21:1981–1991

Castillo UF, Strobel GA, Ford EJ, Hess WM, Porter H, Jensen JB, Albert H, Robison R, Condron MA, Teplow DB, Stevens D, Yaver D (2002) Munumbicins, wide spectrum antibiotics produced by *Streptomyces* NRRI 30562, endophytic on *Kennedia nigriscans*. Microbiology 148:2675–2685.

Castillo U, Harper JK, Strobel GA, Sears J, Alesi K, Ford E, Lin J, Hunter M, Maranta M, Ge H, Yaver D, Jensen JB, Porter H, Porter H, Robison R, Millar D, Hess WM, Condron M, Teplow D (2003) Kakadumycins, novel antibiotics from *Streptomyces* sp. NRRI 30566, an endophyte of *Grevillea pteridifolia*. FEMS Lett 224:183–190

Daisy B, Strobel G, Ezra D, Castillo U, Hess WM (2002a) *Muscodor vitigenus* anam sp. Nov., an endophyte from *Paullinia paulliniodes*. Mycotaxon 84:39–50

Daisy BH, Strobel GA, Castillo U, Ezra D, Sears J, Weaver D, Runyon JB (2002b) Napthalene, an insect repellent is produced by *Muscodor vitigenus*, a novel endophytic fungus. Microbiology 148:3737–3741

Deshmukh S, Huckelhoven R, Schafer P, Imani J, Sharma M, Weiss M, Waller F, Kogel K-H (2006) The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. Proc Natl Acad Sci USA 103:18450–18457

Dreyfuss MM, Chapela IH (1994) Potential of fungi in the discovery of novel, low molecular weight pharmaceuticals. In: Gullo VP (ed) The discovery of natural products with therapeutic potential. Butterworth-Heinemann, London, pp 49–80

Findlay JA, Bethelezi S, Li G, Sevek M (1997) Insect toxinsfrom an endophyte fungus from wintergreen. J Nat Product 60:1214–1215

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Giovannetti M, Sbrana C (1998) Meeting a non-host: the behaviour of AM fungi. Mycorrhiza 8:123–130

Hawksworth DC, Rossmann AY (1987) Where are the undescribed fungi? Phytopathology 87:888–891

Hazarika BN (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704–1712

Hill TW, Käefer E (2001) Improved protocols for *Aspergillus* minimal medium: trace element and minimal medium salt stock solutions. Fungal Genet Newslett 48:20–21

Horn WS, Simmonds MSJ, Schwartz RE, Blaney WM (1995) Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. Tetrahedron 14:3969–3978.

Kaldorf M, Koch B, Rexer K-H, Kost G, Varma A (2005) Patterns of interaction between *Populus* Esch5 and *Piriformospora indica*: a transition from mutualism to antagonism. Plant Biol 7:210–218

Kumari R, Yadav HK, Bhoon YK, Varma A (2003) Colonization of Cruciferous plants by *Piriformospora indica*. Curr Sci 85:1672–1674

Kumari R, Pham GH Sachdev M, Garg AP, Varma A (2004) Symbiotic fungi for eco-friendly environment: a perspective. natural product radiance. CSIR 3:396–400

Li JY, Strobel GA (2001) Jesterone and hydroxy-jesterone anti-oomycetcyclohexenenone epoxides from the endophytic fungus *Pestalotiopsis jesteri*. Phytochemistry 57:261–265

Lorang JM, Tuori RP, Martnez JP, Sawyer TL, Redman RS, Rollins RS, Wolpert JA, Johnson KB, Rodriguez RJ, Dickman MB, Ciuffetti LM (2001) Green fluorescent protein is lighting up fungal biology. Appl Environ Microbiol 67:1987–1994.

Lu H, Zou WX, Meng JC, Hu J, Tan RX (2000) New metabolites produced by *Colletrotrichum* sp., an endophytic fungus in *Artemesia annua*. Plant Sci 151:67–73

Malla R, Prasad R, Kumari R, Giang PH, Pokharel U, Oelmüeller R, Varma A (2005) Phosphorus solubilizing symbiotic fungus: *Piriformospra indica*. Endocytobiosis Cell Res 15:579–600

Marquez LM, Redman RS, Rodriguez RJ, Roossinck MJ (2007). A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. Science 315:513–515

Matsushima R, Fukao Y, Nishimura M, Hara-Nishimura I (2004) *NAI*1 gene that encodes a basic-helix-loop-helix-type putative transcription factor that regulates the formation of a novel ER-derived structure, the ER body. Plant Cell 16:1536–1549

Mittermeier RA, Meyers N, Gil PR, Mittermeier CG (1999) Hotspots: Earth's biologically richest and most endangered ecoregions. Topan, Tokyo

Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H (2005) The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. Proc Natl Acad Sci USA 102:16919–16926

Oelmüller R, Shahollari B, Peškan-Berghöfer T, Trebicka A, Giang PH, Sherameti I, Oudhoff M, Venus Y, Altschmied L, Varma A (2004) Molecular analyses of the interaction between *Arabidopsis* roots and the growth-promoting fungus *Piriformospora indica*. Endocytobios Cell Res 15:504–517

Oelmüller R, Peškan-Berghöfer T, Shahollari B, Trebicka A, Sherameti I, Varma A (2005) MATH-domain proteins represent a novel protein family in *Arabidopsis thaliana* and at least one member is modified in roots in the course of a plant/microbe interaction. Physiol Plant 124:152–166

Peškan-Berghöfer T, Shahollari B, Giang PH, Hehl S, Markent C, Blank V, Kost G, Varma A, Oelmueller R (2004) Association of *Piriformospora indica* with *Arabidopsis thaliana* roots represent a novel system to study beneficial plant-microbe interactions and involve in early plant protein modifications in the endocytoplasmic reticulum and in the plasma membrane. Plant Physiol 122:465–471

Pham GH, Singh A, Malla R, Kumari R, Prasad R, Sachdev M, Luis P, Kaldorf M, Tatjana P, Harrmann S, Hehl S, Declerck S, Buscot F, Oelmüller R, Rexer KH, Kost G, Varma A (2004a) Interaction of *P. indica* with other microorganisms and plants. In: Varma A, Abbott L, Werner D and Hampp R (eds) Plant surface microbiology. Springer, Heidelberg, pp 237–265

Pham GH, Kumari R, Singh A, Sachdev M, Prasad R, Kaldorf M, Buscot F, Oelmüller R, Tatjana P, Weiss M, Hampp R, Varma A (2004b) Axenic cultures of *Piriformospora indica*. In: Varma A, Abbott L, Werner D and Hampp R (eds) Plant surface microbiology. Springer, Heidelberg, pp 593–616

Pham GH, Srivastava A, Saxena AK, Pareek A, Varma A (2004c) Protocol to understand the interaction between rhizobacteria and symbiotic fungus: *Piriformospora indica*. In: Podila G, Varma A (eds) Basic research and applications: mycorrhizae. Microbiology series, vol 1. IK International, India, pp 425–450

Rai M, Varma A (2005) Arbuscular mycorrhiza-like biotechnological potential of *Piriformospora indica*, which promotes the growth of *Adhatoda vasica* Nees. Electron J Biotechnol 8:107–112.

Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation by *Piriformospora indica* in a field trial. Mycorrhiza 11:123–128.

Rai MK, Varma A, Pandey AK (2004) Enhancement of antimycotic potential in *Spilanthes calva* after inoculation of *Piriformospora indica*, a new growth promoter. Mycoses 47:479–481

Redell P, Gordon V (2000) Lessons from nature: can ecology provide new leads in the search for novel bioactive chemicals from rainforests? In: Wrigley SK, Hayes MA, Thomas R, Chrystal EJT and Nicholson N (eds). Biodiversity: new leads for pharmaceutical and agrochemical industries. The Royal Society of Chemistry, Cambridge, UK, pp 205–212

Redman RS, Ranson JC, Rodriguez RJ (1999) Conversion of the pathogenic fungus Colletotrichum magna to a nonpathogenic, endophytic mutualist by gene disruption. Mol Plant Microbe Interact. 12:969–975.

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance generated by plant/fungal symbiosis. Science 298:1581

Rodriguez R, Redman R (2001) Balancing the generation and elimination of reactive oxygen species. Proc Natl Acad Sci USA 102:3175–3176

Rodriguez RJ, Redman RS, Henson JM. (2004a) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitigation Adapt Strategies Global Change 9:261–272

Rodriguez RJ, Redman RS, Henson JM (2004b) Symbiotic lifestyle expression by fungal endophytes and the adaptation of plants to stress: unraveling the complexities of intimacy. In: Dighton J, Oudemans P, White J (eds) The fungal community: its organization and role in the ecosystem. Dekker, New York, pp 683–695

Sahay NS, Varma A (1999) *Piriformospora indica*: a new biological hardening tool for micropropagated plants. FEMS Microbiol Lett. 181:297–302.

Sahay NS, Varma A (2000) Biological approach towards increasing the survival rates of micropropagated plants. Curr Sci 78:126–129

Shahollari B, Varma A, Oelmüeller R (2005) Expression of a receptor kinase in Arabidopsis roots is stimulated by the basidiomycete *Piriformospora indica* and the protein accumulates in Triton X-100 insoluble plasma membrane microdomains. J Plant Physiol 162:945–958

Shahollari B, Vadassery J, Varma A, Oelmüller R (2007) leucine-rich repeat protein is required for growth promotion and enhanced seed production mediated by the endophytic fungus *Piriformospora indica* in *Arabidopsis thaliana*. Plant J 50:1–13(13)

Sherameti I, Shahollari B, Venus Y, Altschmied L, Varma A, Oelmüller R (2005) The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain transcription factor that binds to a conserved motif in their promoters. J Biol Chem 280:26241–26247

Sherameti I, Tripathi S, Varma A, Oelmüller R (2007a) The root colonizing endophyte *Pirifomospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of a diverse set of drought-stress related genes in leaves. Mol Plant Microbe Int (in press)

Sherameti I, Venus Y, Drezewiecki C, Tripathi S, Dan VM, Grundler FM, Varma A, Oelmüller R (2008a) PYK10, a â-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between *Arabidopsis thaliana* and the endophytic fungus *Piriformospora indica*. Plant J 54:428–439

Singh An, Singh A, Malla R, Ghosh S, Varma A (2002) Symbiotic fungi: a boon for plant industry. Conference on Biotechnology – the Science & the Business. IIT, AIBA, New Delhi, pp 52–55

Singh An, Singh A, Kumari M, Kumar S, Rai MK, Sharma AP, Varma A (2003a) AMF-like-fungus: *Piriformospora indica* – a boon for plant industry. In: Prasad BN (ed) Biotechnology in sustainable biodiversity and food security. Science Publishers, Enfield, N.H., pp 101–124

Singh An, Singh A, Kumari M, Rai MK, Varma, A (2003b) Biotechnology importance of *Piriformospora indica* – a novel symbiotic mycorrhiza-like fungus: an overview. Ind J Biotechnol 2:65–75

Singh Ar, Singh A, Varma A (2002) *Piriformospora indica – in vitro* raised leguminous plants: a new dimension in establishment and phyto-promotion. Ind J Biotechnol 1:372–376

Siqueira JO, Safir GR, Nair MG (1991) Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. New Phytol 118:87–93

Strobel GA, Li JY, Sugawara F, Koshino H, Harper J, Hess WM (1999) Oocydin A, a chlorinated macrocyclic lactone with potent anti-oomycete activity from *Serratia marcescens*. Microbiology 145:3557–3564

Strobel G, Daisy B (2003) Bioprespecting for microbial endophytes and their natural products. Mcrobiol Mol Biol Rev 7:491–502

Strobel GA, Dirskie E, Sears J, Markworth C (2001) volatile antimicrobials from a novel endophytic fungus. Microbiology 147:2943–2950

Varma A, Verma S, Sudha, Sahay N, Britta B, Franken P (1999) *Piriformospora indica* – a cultivable plant growth promoting root endophyte with similarities to arbuscular mycorrhizal fungi. Appl Env Microbiol. 65:2741–2744

Varma A, Rai MK, Sudha, Sahay N (2000) Microbial-biotechnology: new-paradigms and role in sustainable agriculture. In: Rajak RC (ed) Microbial biotechnology for sustainable development and productivity. Scientific Publishers (India), pp 22–37

Varma A, Singh A, Sudha, Sahay N, Sharma J, Roy A, Kumari M, Rana D, Thakran S, Deka D, Bharti K, Franken P, Hurek T, Blechert O, Rexer K-H, Kost G, Hahn A, Hock B, Maier W, Walter M, Strack D, Kranner I (2001) *Piriformospora indica*: a cultivable mycorrhiza-like endosymbiotic fungus. In: Hock B (ed) Mycota IX. Springer, Heidelberg, pp 123–150

Verma S, Varma A, Rexer K-H, Hassel A, Kost G, Sarbhoy A, Bisen P, Buetehorn P, Franken P (1998) *Piriformospora indica* gen. nov., a new root-colonizing fungus. Mycologia. 90:895–909

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Hückelhoven R, Neumann C, von Wettstein D, Franken P, Kogel K-H (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Nat Acad Sci USA 102:13386–13391.

Worapong J, Strobel GA, Ford J, Li JY, Baird G, Hess WM (2001) *Muscodor albus* gen. Et sp. Nov. an endophyte from *Cinnamomum Zeylanicum*. Mycotaxon. 79:67–79

Zhang B, Salituro G, Szalkowski D, Li Z, Zhang Y, Royo I, Viella D, Dez M, Pelaez F, Ruby C, Kendall RL, Mao X, Griffin P, Calaycay J, Zierath JR, Heck JV, Smith RG, Moller DE (1999) Discovery of small molecule insulin mimetic with antidiabetic activity in mice. Science 284:974–981

Zou WX, Meng JC, Lu H, Chen GX, Shi GX, Zhang TY, Tan RX (2000) Metabolites of *Colletotrichum gloeosporiodes*, an endophytic fungus in *Artemesia mongolica*. J Nat Product 63:1529–1530

Frankia Nodulation, Mycorrhization and Interactions Between *Frankia* and Mycorrhizal Fungi in *Casuarina* Plants

X. H. He(✉), C. Critchley

1 Introduction

Casuarina, literally "*Kasuari*" from the Malay word for the "cassowary" bird, bears a resemblance of the plant's drooping foliage to the bird's feather (Boland et al. 1994). *Casuarina* has probably evolved since the Tertiary times and looks more like a wispy conifer, since its needle-like foliage is similar in shape to a coniferous needle, and its vegetative and floral parts show considerable scleromorphy (Midgley et al. 1983; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996). The foliage of *Casuarina* plants is not a true leaf, called cladode or cladophyll, but functions like a green leaf. Most *Casuarina* species typically regenerate from their fine seeds, about 400,000–600,000/kg. Seeds are readily extracted from air-dried fruits and germination is epigeal on moist, bare soil. In addition, *Casuarina equisetifolia* can be propagated from cuttings, *C. glauca* is able to spread clonally from root suckers, and *C. junghuhniana* is fire-tolerant and is able to sprout rapidly after fire. Nodulation does not normally form when planted outside the native habitats unless the indigenous *Frankia* is introduced with the *Casuarina* seedlings (Simonet et al. 1999). Some species, such as the native New Guinea's *C. oligodon*, have been planted for more than 3,000 years (Midgley et al. 1983; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996).

Casuarina is currently classified as one of the four genera (*Allocasuarina, Casuarina, Ceuthostoma*, and *Gymnostoma*) in the family *Casuarinaceae*, native plants grown in Australia and islands of the Pacific. There are approximately 18 species in the genus *Casuarina* (Table 1). Of them, *Casuarina cunninghamiana, C. equisetifolia* and *C. glauca* were introduced and planted worldwide, including in Argentina, Bangladesh, Chile, China, Egypt, India, Japan, Kenya, Israel, Mexico, Puerto Rico, South Africa, the Philippines, the United States, Thailand, Tanzania, Zimbabwe and

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Table 1 *Casuarina* species and their geographic distribution

Species scientific name	Common name	Geographic distribution
C. collina Poiss. ex Sebert and Pancher	NA	New Caledonia
C. cristata Miq. (synonym of *C. lepidophloia* F.Muell.)	Belah	Eastern Australia
C. cristata Miq. ssp. *cristata*		Eastern Australia
C. cunninghamiana Miq.	River She-oak	North and Eastern Australia
C. cunninghamiana ssp. *cunninghamiana*		Eastern Australia
C. cunninghamiana ssp. *miodon* L.A.S. Johnson		Northern Australia
C. equisetifolia L.	Coast/Beach She-oak	Australia, New Caledonia, New Hebrides
C. equisetifolia ssp. *equisetifolia* L.		Malesia, Australia, Melanesia, Polynesia
C. equisetifolia ssp. *incana* (Benth.) L.A.S. Johnson		Australia, New Caledonia, New Hebrides
C. glauca Sieber ex Spreng.	Grey /Swamp She-oak	Eastern Australia
C. grandis L.A.S. Johnson	NA	South-eastern New Guinea
C. junghuhniana Miq.	Forest oak	Indonesia
C. junghuhniana ssp. *timorensis*		Indonesia, Timor
C. obesa Miq.	Swamp She-oak	Australia
C. oligodon L.A.S. Johnson	She-oak	New Guinea
C. oligodon ssp. *abbreviate* L.A.S. Johnson		Western New Guinea
C. oligodon ssp. *oligodon* L.A.S. Johnson		New Guinea
C. orophila L.A.S. Johnson	NA	Western New Guinea (high altitude)
C. pauper F. Muell. ex L.A.S. Johnson	Black Oak	Inland Australia
C. sp. 'Aneityum' (L. Bernardi 13045) L.A.S. Johnson ms	NA	New Hebrides
C. sp. 'Celebes' (O. Weismann 340) L.A.S. Johnson ms	NA	Celebes
C. sp. 'Palawan' (C. Ridsdale SMHI1777) L.A.S. Johnson ms	NA	Palawan
C. sp. 'Riparian' (H. Curran FB6993) L.A.S. Johnson ms	NA	Philippines
C. sp. 'Santo' (H. McKee RSNH 24301) L.A.S. Johnson ms	NA	New Hebrides (Espiritu Santo)
C. sp. 'Timor' (F. McKinnell NSW 247142) L.A.S. Johnson ms	NA	Sumba, Timor, Wetar
C. teres Schltr.	NA	New Caledonia

NA Not available

Sources: Wilson and Johnson 1989; Wilson and Berendsohn 2005; Bisby et al. 2007

some European countries, for more than 100 years (Midgley et al. 1983; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996). They are now even treated as invasive species, particularly on the seashores of Puerto Rico, Florida and the Hawaiian Islands, for their fast growth rates with large biomass production and their

possible alleopathic properties to replace the native vegetation. This has been evidenced by less nonwood species richness and diversity and even the absence of understory nonwood vegetation beneath the *Casuarina* canopy (Parrotta 1999; Lugo 2004).

As multipurpose trees, *Casuarina* has wide applications in agroforestry, forestry, land reclamation and silviculture (Midgley et al. 1983; National Research Council 1984; Diem and Dommergues 1990; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996). The dense, hard and relative smokeless wood of *Casuarina* makes it a super-quality fuel and timber wood as well as good industrial charcoal with a considerably higher energy value (4,000–5,000 kcal/kg) than that of other plants (National Academy of Sciences 1980; Barlow 1983; Rockwood et al. 1983; Subba-Rao and Rodriguez-Barrueco 1995). For example, *C. cunninghamiana* and *C. equisetifolia* grow rapidly up to 2–3 m/year when young, and can reach a height of 20–30 m or even up to 50 m, whereas the average height of an 8-year-old *C. glauca* is greater than 10 m (National Academy of Sciences 1980; Goel and Behl 2005).

C. cunninghamiana, C. equisetifolia and *C. glauca* are widely planted in parks and streets as hedges and ornamentals. They are also planted on mining spoil dumps, desert dunes and coastal area for sand/soil stabilization and as windbreaks, since their reduced needles and needle-like branchlets construct a permeable barrier to effectively absorb wind energyy. *C. junghuhniana* is extensively cultivated for marine poles and house foundation supports in Thailand. *C. equisetifolia* associated with cashew (*Anacardium occidentale*) and coconut (*Cocos nucifera*) has been successfully intercropped on the coast of India (Kumar 1981). *C. oligodon* is commonly used for building timber, furniture and tools, and planted as shading or covering plants in the coffee plantation of New Guinea (Midgley et al. 1983). In addition, the gum exuded from some casuarinas is edible and was once a food source for the aboriginal people in Australia. The bark of *C. equisetifolia* has been used in tanning and medicine, and for the extraction of dyes (Olson and Petteys 1974).

Casuarina has also been substantially used as an effective soil improver in nutrient-deficient soils by its nitrogen (N)-fixation capacity to increase soil fertility (Midgley et al. 1983; National Research Council 1984; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996; Forrester et al. 2006). Percentage of N_2-fixation in *Casuarina* species ranges from 15 to 80% of N derived from atmospheric (% Ndfa) (Sougoufara et al. 1989; Sanginga et al. 1990; Subba-Rao and Rodriguez-Barrueco 1995), comparable to leguminous plants (Mariotti et al. 1992; Dommergues 1995; Parrotta et al. 1999; Swensen 1996; Sprent and Parsons 2000). Along with *Acacia* spp. and *Prosopis* spp., *Casuarina* is considered to be one of the most valuable multipurpose nonleguminous N_2-fixing perennials in the tropics and subtropics around the world.

In addition, *Casuarina* root systems can associate with either arbuscular mycorrhizal (AM) fungi, ectomycorrhizal (EM) fungi or both to form mycorrhiza (Warcup 1980; Gauthier et al. 1983; Gardner 1986; Reddell et al. 1986; Theodorou and Reddell 1991; Vasanthakrishna and Bagyaraj 1993; Vasanthakrishna et al. 1994a, 1994b, 1995; Subba-Rao and Rodriguez-Barrueco 1995; Reddell et al. 1997; Osundina

1998; Singh et al. 1998; Mark et al. 1999; Wang and Qiu 2006), which helps their host plants to access soil nutrients and water. Some *Casuarina* species, including *C. cunninghamiana, C. equisetifolia* and *C. glauca*, even have the capacity to form cluster roots, which specialize in phosphorus (P) uptake from the soil (Diem 1996; Arahou and Diem 1997; Reddell et al. 1997; Diem et al. 2000; Zaïd et al. 2003; Lambers et al. 2006). In addition, a greater soil carbon (C) sequestration under *C. equisetifolia* than under *Eucalyptus* trees may provide important reforestation or afforestation strategies to compensate C emissions (Resh et al. 2002), particularly in times of global environmental change.

2 *Frankia* Nodulation

All *Casuarina* species are able to convert atmospheric N_2 gas into ammonia (N_2-fixation) by the Gram-positive actinomycete *Frankia*, a filamentous, aerobic bacterium, in root nodules (National Research Council 1984; Diem and Dommergues 1990; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et a. 1996). *Frankia* was named in honor of Albert Bernard Frank, a pioneer who studied symbiosis and coined the term "mycorrhiza" about 120 years ago (Frank 1888; see Gardner 1986). *Frankia* belongs to the Order Actinomycetales and the Kingdom Bacteria, and is able to nodulate a diverse range of flowering plants that are therefore termed actinorhizal (nonleguminous) plants (Baker and Schwintzer 1990; Franche et al. 1998; Wall 2000; Schwencke and Carú M 2001). These plants belong to nearly 200 species of 25 genera in 8 angiosperm families (Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae and Rosaceae) (Benson and Silvester 1993; Wall 2000).

Numerous *Frankia* strains have been currently identified after its first isolation obtained in vitro in the late 1970s (Callaham et al. 1978). This is much later than the Gram-negative rhizobia isolated in the root nodules of leguminous plants at the end of the nineteenth century (Sprent and Sprent 1990). In general, the *Frankia* endophyte exhibits a distinct polymorphism both in the nodule and in the free-living state, and the growth of *Frankia* is slow and pleomorphic (Schwencke and Carú 2001).

Most *Frankia* strains present three typical characteristics: branched and septate hyphae, NIR (nitrogen-reducing) vesicles, and multilocular sporangia (Torrey and Callaham 1982; Lechevalier and Lechevalier 1990; Benson and Silvester 1993; Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003), depending on the conditions of the culture (Newcomb and Wood 1987). The presence of "torulose hyphae" has also been reported in *Casuarina* (Diem and Dommergues 1985). *Frankia* is able to form NIR-vesicles in culture, but no recognizable NIR-vesicles have been observed in *Casuarina* root nodules. Sporangia are formed through the swelling of *Frankia* hyphae in the late growth phase, and the ultrastructures of sporangia have been observed in strains isolated from *Casuarina* culture (Lancelle et al. 1985). Sporangia then release numerous irregularly-shaped nonmotile spores when mature.

The nodule formation is initiated through root hair or intracellular infection by *Frankia* (Berry and Sunnel 1990). Root hair infection is induced through hair curling by an unknown *Frankia* signal, and the curled root hairs are penetrated by *Frankia* (Obertello et al. 2003). The penetration of hyphae occurs at the base of a deformed root hair in *C. cunninghamiana* (Torrey 1976) and the infection is carried out intracellularly in the root cortex (Obertello et al. 2003). After penetration, *Frankia* hyphae are then encapsulated by a cell wall material containing cellulose, hemicellulose and pectins (Berg 1990). In response to the initial root hair infection, a small protuberance, the prenodule, is formed near the invaded root hair through cell division occurring in the cortex (Berry and Sunnel 1990).

As the prenodule develops, cell divisions are induced in the pericycle opposite the protoxylem pole and given rise to the nodule primordium. The primordia traverse the cortical tissue in the midst of the infected prenodule cells in *C. cunninghamiana* (Torrey 1976), while developing outside the infected prenodule tissue in *C. glauca* (Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003). As the primordium grows, the nodule develops from the mitotic activity in pericycle cells (Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003). The mature nodule consists of multiple lobes shaped as modified lateral roots (Obertello et al. 2003). The prenodule could thus act as a nodule precursor (Laplaze et al. 2000). An *ENOD40* (early nodulin) gene promoter is induced during nodule occurrence in *C. glauca*, but has not been observed in other *Casuarina* plants (Santi et al. 2003).

Frankia nif (nitrogen-fixation) genes are expressed and the nitrogenase is then activated to fix N_2 in the nodule. In doing so, two elements of Fe and Mo are required for the proper function of the nitrogenase. A protein, called metallothionein, provides these two elements for the biosynthesis of the Fe-Mo cofactor of the nitrogenase complex, and is involved in elemental homeostasis and cell differentiation. A cDNA corresponding to a nodule-enhanced gene of metallothionein in *C. glauca, cgMT1* is expressed in the infected cells. The presence of *cgMT1* transcripts in the pericycle suggests that the pericycle is also involved in metal exchanges in *C. glauca* (Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003). In addition, the expression of *cgMT1* in *C. glauca* nodules seems to be correlated with the expression of a *nifH* gene (the gene encoding nitrogenase reductase), which appears as an indication for bacterial N_2-fixation, since the product of *nifH* gene is a common marker for the nitrogenase reductase (Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003).

On the one hand, oxygen is needed for respiration of the infected cells. On the other hand, nitrogenase, an oxygen-sensitive enzyme, is also inhibited or even destroyed by traces of oxygen. Fortunately, the lignification of infected cell walls may prevent oxygen diffusion (Berg and McDowell, 1988), and/or a hemoglobin (Hb) allows a low oxygen affinity in *Casuarina* nodules (Fleming et al. 1987; Silvester et al. 1990). In addition, *Casuarina hb* genes are expressed before *nif* genes (see Franche et al. 1998). In situ hybridization showed that *hb* mRNAs were concentrated in the mature infected cells of the cortical tissues, whereas the induction of *nifH* of *Frankia* occurred in *C. glauca* nodules (Gherbi et al. 1997). Meanwhile, the expression of Hb of *C. glauca* was similar between prenodule- and

nodule-infected cells, suggesting N_2-fixation may already occur in the prenodule stage (Gherbi et al. 1997; Franche et al. 1998; Schwencke and Carú 2001; Obertello et al. 2003).

Furthermore, the membranes of *Frankia* contain amphiphilic, pentacylic triterpenoid lipids, called hopanoids. Hopanoids are the major compounds of the extracellular envelope of *Frankia* vesicles (Berry et al. 1993). The N_2-fixation enzyme, nitrogenase, is located in the *Frankia* vesicle where the conversion of atmospheric dinitrogen to ammonium occurs. The hopanoids are able to thicken and stabilize the membranes and thus help to keep the nitrogenase away from oxygen, whilst the *Frankia* vesicle provides inducible and absolute oxygen protection for nitrogenase (Parsons et al. 1987). All these mechanisms could provide a balanced oxygen requirement for a proper N_2-fixation. The product of N_2-fixation, ammonium, is exported by *Frankia* to the plant cell cytoplasm, where it is assimilated through the GS/GOGAT pathway and is then metabolized to synthesize or transform into various nitrogen-containing compounds (Schubert 1986).

The growth of *Casuarina* is substantially enhanced by inoculating appropriate *Frankia* strains. For example, plant height and wood production of *C. cunninghamiana* were increased by 1.5–3 and 2 times, respectively, (Reddell et al. 1988; Reddell 1990), and biomass production of *C. equisetifolia* was respectively increased by 45, 36 and 40% at 1, 2 and 3 years after transplanting (Sougoufara et al. 1989). Root nodule formation was substantially affected by abiotic factors including nutrient availability of N (Kohls and Baker 1989; Arnone et al. 1994) and P (Sanginga et al. 1989; Yang 1995) and other soil properties (Dawson et al. 1989). A positive synergism between *Frankia* inoculum and soil biota has also been achieved in *C. cunninghamiana* (Zimpfer et al. 2003). Compared to the nonnodulated plants, more nodules, greater nodule dry materials and nitrogenase activity were obtained in nodulated *C. equisetifolia* with *Frankia* strain CcI3 (Shah et al. 2006).

3 Mycorrhization

In addition to *Frankia* symbiosis, *Casuarina* plants can form another symbiotic association with either AM or EM fungi (Rose 1980; Gardner 1986; Sidhu et al. 1990; Cervantes and Rodriguez-Barrueco 1992; Theodorou and Reddell 1991; Khan 1993; Subba-Rao and Rodriguez-Barrueco 1995; Smith and Read 1997). A variety of AM or EM fungal species is given in Table 2. In general, either AM or EM could contribute to a successful *Frankia* symbiosis in *Casuarina* plants by increasing N_2-fixation especially in P-deficient soils, by enhancing uptake of P, other mineral nutrients and water from the soil, and by having a generally protective role against environmental stresses, such as in arid climates (Gardner 1986; Theodorou and Reddell 1991; Subba-Rao and Rodriguez-Barrueco 1995; Smith and Read 1997). An important physiological and ecological distinction between AM and EM is that AM develops more extensively in mineral soils whereas EM flourishes in highly organic surface soils (Smith and Read 1997). In general, EM

Table 2 Known species of AM and EM fungi capable of forming mycorrhizas in *Casuarina* plants

AM fungal species	EM fungal species
Acaulospora laevis Gerdemann and Trappe	*Amanita* sp.
Glomus albidum Walker and Rhodes	*Elaphomyces* sp.
G. claroideum Schenck and Smith	*Hysterangium* sp.
G. clarum Nicolson and Schenck	*Laccaria laccata* (Scop ex Fr.) Bk. and Br.
G. fascicultum (Thaxter Sensu Gerdemann) Gerdemann and Trappe	*Paxillus involutus* (Batch ex Fr.) Fr.
G. geosporum (Nicolson and Gerdemann) Walker	*P. albus* IR100 Bougher and Smith
G. intraradices Schenck and Smith	*Pisolithus tinctorius* (Pers.) Coker and Couch
G. macrocarpum (Nicolson and Gerdemann) Gerdemann and Trappe	*Rhizopogon luteolus* Fr. and Nord
G. monosporum Gerdemann and Trappe	*Scleroderma* sp.
G. mosseae (Nicolson and Gerdemann) Gerdemann and Trappe	*Suillus granulatus* (L. ex Fr.) Kuntze
G. rubiforme (Gerdemann and Trappe) Almeida and Schenck	*S. piperatus* (Bull ex Fr.) O. Kuntze
G. versiforme (Karsten) Berch	*Thelephora terrestris* (Ehrh.) Fr.
Gigaspora margarita Gerdemann and Trappe	
Scutellospora sp.	

Sources: Rose 1980; Gardner1986; Theodorou and Reddell 1991; Vasanthakrishna and Bagyaraj 1993; Vasanthakrishna et al. 1995

association is more popular in *Casuarina* species growing in drier environments (Torrey 1983; Subba-Rao and Rodriguez-Barrueco 1995; Smith and Read 1997). However, *Casuarina* plants in the Libyan and Egyptian deserts are probably exclusive AM (El-Giahmi et al. 1976; Gardner 1986). Dual AM and EM have also been found in *Casuarina* species, particularly in *C. cunninghamiana* and *C. equisetifolia* (Reddell et al. 1986; Ba et al. 1987; Cervantes and Rodriguez-Barrueco 1992; Singh et al. 1998; Wang and Qiu 2006). On the other hand, differences exist among *Casuarina* species in forming mycorrhizas with the same fungal species, whereas mycorrhiza-forming ability also varies among fungal isolates of the same fungal species with the same *Casuarina* species (Thoen et al. 1990).

Arbuscular mycorrhizal (AM) colonization ranges from 10 to 70% while EM ranges from 10 to 50% in *Casuarina* plants (Gardner 1986; Theodorou and Reddell 1991; Vasanthakrishna and Bagyaraj 1993; Subba-Rao and Rodriguez-Barrueco, 1995; Vasanthakrishna et al. 1995; Singh et al. 1998; He et al. 2004, 2005). The effectiveness and AM colonization through various AM species are consistent with different *Casuarina* species. In general, percentage of AM colonization in *Casuarina* seedlings was greater in the mycorrhizal-nodulated treatment than in the mycorrhizal nonnodulated treatment (Gardner 1986). However, the effectiveness and mycorrhizal colonization are variable with both mycorrhizal fungal and plant species. For example, two isolates of *Pisolithus* (H4111) and *Scleroderm* (H4314) could only induce a very low (1.7–2.3%) root colonization in 3-month-old *C. equisetifolia* roots (Dell et al. 1994). In the same experiment, another isolate of

Pisolithus (H4458) was unable to form EMs and the *Scleroderm* (H4151) isolate has even resulted in the death of *C. equisetifolia* seedlings (Dell et al. 1994). In contrast, Duponnois et al. (2003) recently found that *Pisolithus* and *Scleroderina* isolates did form EMs on *C. glauca* under axenic conditions, but without the appearance of the typical Hartig net. Furthermore, EM colonisation was similar between 6-month-old and 12-month-old *Casuarina*, no matter which were dual mycorrhizal-nodulated or mycorrhizal alone (He et al. 2004, 2005).

Mycorrhizal colonization and spore numbers were greater in the root zone soil (Vasanthakrishna and Bagyaraj 1993; Vasanthakrishna et al. 1995). Inoculated *C. equisetifolia* plants displayed greater plant height, biomass, and P and Zn contents. Among the 13 AM species tested, *Glomus mosseae, G. fasciculatum, G. versiforme* and *Acaulospora laevis* were the most effective (Vasanthakrishna and Bagyaraj 1993; Vasanthakrishna et al. 1995). Compared to the nonmycorrhizal plants, a recent study showed that nodules, nodule dry materials and nitrogenase activity were greatly increased in *C. equisetifolia* after inoculated with AM of *G. fascicultum* (Shah et al. 2006).

The relative importance of AM and EM associations in *Casuarina* plants was recently investigated (Duponnois et al. 2003). Plant growth of *C. glauca* was stimulated by an AM *Glomus intraradices* or an EM *Pisolithus albus* in a controlled soil system. Antagonism occurred between AM and EM fungal species. A higher AM colonization was observed and the response of plant growth to AM inoculation was greater than with EM inoculation, indicating *C. glauca* was AM dependent. This antagonistic interaction between AM and EM in *C. glauca* demonstrated the requirement for further investigation of AM and EM effects on the function of N_2-fixation actinorhizal plants (Duponnois et al. 2003).

4 Interactions of *Frankia* and Mycorrhizal Fungi

The interaction among actinorhizal plants, mycorrhizas, and *Frankia* is a complex, multilayered relationship. These three organisms interact and impact each other for an apparent *Casuarina* performance. Numerous studies have demonstrated that the percentage of root mycorrhizal colonisation in *Casuarina* plants is generally greater in the dual mycorrhizal-nodulated treatment than in the single mycorrhizal treatment (Midgley et al. 1983; Gardner 1986; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996). The dual AM mycorrhizal-nodulated *Casuarina* plants exhibited better overall growth, enhanced P uptake and better nodulation and N_2-fixation than did the nodulated-only *C. equisetifolia* (Gauthier et al. 1983). Greater nodulation activity and plant growth of *C. equisetifolia* were enhanced wwith dual inoculation with both *G. fasciculatum* and *Frankia* than with the single inoculation with either *G. fasciculatum* or *Frankia* (Vasanthakrishna et al. 1994a). The effects of *G. fasciculatum* were even greater than those of *Frankia* (Vasanthakrishna et al. 1994a). The greatest growth of shoot and roots and the total biomass yield were also increased in the dual mycorrhizal (*G. fasciculatum*)-nodulated *C. equisetifolia*

(Shah et al. 2006). AM-inoculated *C. equisetifolia* seedlings performed better than the nonmycorrhizal ones under an 8-week flooding anoxic condition, by greater development of adventitious roots and hypertrophied lenticels for greater oxygen availability and less accumulation of anaerobic respiration products of toxic ethanol (Osundina 1998).

Meanwhile, no significant difference in the overall growth was found between 3-month-old dual mycorrhizal-nodulated and nonmycorrhizal-nodulated *C. equisetifolia*, although both nodulated treatments displayed significantly greater growth than the mycorrhizal nonnodulated and the nonmycorrhizal noninoculated ones (Gardner 1986). The formation of nodules in the mycorrhizal-nodulated plants was twice as efficient as those formed on the nonmycorrhizal-nodulated plants. However, nodulation was inhibited in the mycorrhizal-nodulated plants, indicating a longer growth period would be required for a full nodulation and mycorrhizal development, while the drain on plant photosynthates by the two symbionts is comparatively stronger, particularly in the early growth stage (Gardner 1986). It may also be possible that competition exists between *Frankia* and mycorrhizal fungi for infection sites or nutrient supply. In addition, the nodule initiation and nodulation in the N_2-fixation process is stimulated by a high P requirement in *Casuarina* plants (Sanginga et al. 1989; Subba-Rao and Rodriguez-Barrueco 1995), whereas the major function of mycorrhizas is to enhance nutrient uptake, particular P by AM (Jasper et al. 1979; Smith and Read 1997). As a consequence, a better plant performance could be promoted by an active *Frankia* N_2-fixation with the proper P supply through a mycorrhizal-enhanced P uptake.

A greater seedling height, girth at breast height, nodule numbers and biomass, plant total biomass and nutrient uptake (N, P, K, Ca and Mg) was respectively patterned by the following treatments: the quadric-inoculation Azospirillum/Phosphobacteriurn/AM/Frankia > the triple Azospirillum/AM/Frankia > the double AM/Frankia > the single Azospirillum, Phosphobacteriurn, AM or Frankia (Rajendran et al. 2004; Rajendran and Devaraj 2004). In addition, the quadric-inoculation Azospirillum/Phosphobacteriurn/AM/Frankia treatment contributed the maximum nutrient return through litter in the field condition (Rajendran et al. 2004; Rajendran and Devaraj 2004).

Under limited supply of mineral N and P, up to 50% lower AM mycorrhizal colonization was detected by a 60-day delay with *Frankia* in the *Glomus* inoculated seedlings or with *Glomus* in the *Frankia* inoculated seedlings, though such delayed inoculation with either *Glomus* or *Frankia* had no long-term impact on subsequent nodulation (Sempavalan et al. 1995). Meanwhile, neither the development of nodules nor mycorrhizal colonization was delayed in the AM-nodulated *C. equisetifolia* seedlings when they were respectively preinoculated with *Glomus* or *Frankia* for a period of 60 days. In addition, a highly linear relationship between the nodule numbers or the % *Glomus* root colonization and the days after *Frankia* or *Glomus* inoculation suggested a high degree of coordination between nodulation, mycorrhization and seedling growth. A cooperative, not a competitive interaction between *Frankia* and *Glomus* for nodulation and mycorrhizal colonization of *C. equisetifolia* has therefore been proposed (Sempavalan et al. 1995).

The effect of interactions between *Casuarina* species, *Frankia* and AM on ^{15}N isotope fractionation was investigated under mineral N-deficient and organic-N free conditions (Wheeler et al. 2000). Four species of *C. cunninghamiana, C. equisetifolia, C. glauca*, and *C. junghuniana* were inoculated singly with three *Frankia* strains or were dual inoculated with *Frankia* plus *Glomus fasciculatum*. As a general rule, nodule biomass, N_2-fixation and plant growth were greater when inoculated with *Frankia* + *Glomus* than with *Frankia* or *Glomus* alone (Wheeler et al. 2000). The results also demonstrated a significant linear relationship between cladode N and $\delta^{15}N$ in all four *Casuarina* species when they were inoculated with *Frankia* alone or together with *G. fasciculatum*. However, both %N and $\delta^{15}N$ were similar among *Casuarina* species, no matter which single or dual inoculation was adopted.

The EM (*Pisolithus* sp.)-nodulated *C. cunninghamiana* plants did also have both greater biomass and total N contents than the non-EM-nodulated counterparts, but no statistically significant differences of its N_2-fixation capacity were displayed (He et al. 2004, 2005). With an environmental scanning electron microscope, living hyphae of *Pisolithus* sp. were observed to interconnect *C. cunninghamiana* and *Eucalyptus maculata* in a pot study (He et al. 2004). Through this common EM network, mycorrhizal-mediated two-way N transfer between *Casuarina* and *Eucalyptus* was then investigated by ^{15}N labeling to either plant species acting as N donor, respectively (He et al. 2004). N transfer rates were patterned as follows: the nonmycorrhizal-nonnodulated < the mycorrhizal-nonnodulated < the mycorrhizal-nodulated treatment. Amount of N transferred was generally greater from *Casuarina* to *Eucalyptus* than from *Eucalyptus* to *Casuarina*. The highest amount of N transferred to either *Casuarina* or *Eucalyptus* was achieved by the presence of both *Pisolithus* sp. and *Frankia* (He et al. 2004; 2005). The increased growth of recipients indicated that either *Casuarina* or *Eucalyptus* benefited physiologically from mycorrhizal-mediated nutrient transfer. These results suggested that mycorrhization, together with N availability, plays a vital role in N redistribution, resulting in resource sharing or facilitation between plants.

5 Conclusion

The environmental and economic importance of N_2-fixation *Casuarina* plants is obvious for the improvement of soil fertility and for the optimization of the afforestation or plantation management for sustainable productivity. The multipurpose *Casuarina* plants can contribute N to soil and thus indirectly to enhance the growth of other economically important non-N_2-fixation trees when planted in mixed populations (Midgley et al. 1983; Subba-Rao and Rodriguez-Barrueco 1995; Pinyopusarerk et al. 1996; Attiwill and Adams 1996; Forrester et al. 2006). For example, total biomass of non-N_2-fixation *Eucalyptus robusta* was greater when planted with N_2-fixation *C. equisetifolia* than planted alone (Dommergues and Subba-Rao 2000). More importantly, *Casuarina* plantations, together with other

non-N_2-fixation tree plantations, could provide large production and excellent quality of industrial timber or firewood, and thus compensate for the dramatic losses of native forests and the emission of atmospheric CO_2 which may become crucial in a period of rapid urbanization, industrialization and globalization.

In the past century, there has been great progress in ecology, physiology, cellular and molecular biology of *Casuarina* plants and their control by environmental factors. However, relatively little is known about the mechanisms involved in the belowground symbiotic interaction with either *Frankia* and/or mycorrhizal fungi, compared with that of the much wider attention paid to the rhizobial/mycorrhizal leguminous plants. For all that has been mentioned above, more research is warranted for a better understanding of the interaction among mycorrhizal fungi, *Frankia* and the host plants, and the sustainable multiple-applications of nonleguminous actinorhizal *Casuarina* plants in agriculture, forestry, industry and landscape, particularly in times of global environmental change.

References

Arahou M, Diem HG (1997) Iron deficiency induces cluster (proteoid) root formation in *Casuarina glauca*. Plant Soil 196:71–79

Arnone JA, Kohls SJ, Baker DD (1994) Nitrate effects on nodulation and nitrogenase activity of actinorhizal Casuarina studied in split-root systems. Soil Bio Biochem 26:599–606

Attiwill PM, Adams MA (1996) Nutrition of Eucalypts. CSIRO, Melbourne, Australia

Ba AM, Sougoufara B, Thoen D (1987). The triple symbiosis of *Casuarina equisetifolia* in Senegal. In: Sylvia DM, Hung LL, Graham JH (eds) Mycorrhizae in the next decade: practical applications and research priorities. University of Florida, Gainsville, Fla

Baker DD, Schwintzer CR (1990) Introduction. In: Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic, New York, pp 157–176

Barlow BA (1983) *Casuarina*-a taxonomic and biogeographic review. In: Midgley SJ, Turnbull JW, Johnston RD (eds) *Casuarina*: ecology, management and utilization, CSIRO Forest Research, Melbourne, Australia, pp 17–21

Benson D, Silvester AW (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev 57:293–319

Berg RH (1990) Cellulose and xylans in the interface capsule in symbiotic cells of actinorhizae. Protoplasma 159:35–43

Berg RH, McDowell L (1988) Cytochemistry of the wall of infected cells in *Casuarina* actinorhizae. Can J Bot 66:2038–2047

Berry AM, Sunell LA (1990) The infection process and nodule development. In: Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic, New York pp 61–81

Berry AM, Harriot OT, Moreau RA, Osman SF, Benson DR, Jones AD (1993) Hopanoid lipids compose the *Frankia* visicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proc Natl Acad Sci USA 90:6091–6094

Bisby FA, Roskov YR, Ruggiero MA, Orrell TM, Paglinawan LE, Brewer PW, Bailly N, van Hertum J (2007) Species 2000 and ITIS catalogue of life: 2007 annual checklist. Digital resource at www.catalogueoflife.org/annual-checklist/2007/. Species 2000, Reading, UK

Boland DJ, Brooker MIH, Chippendale GM, Hall N, Hyland BPM, Johnston RD, Kleinig DA, Turner JD (1994) Forest trees of Australia. CSIRO, Melbourne, Australia

Callaham D, del Tredici P, Torrey JG (1978) Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. Science 199:899–902

Cervantes E, Rodriguez-Barrueco C (1992) Relationships between the mycorrhizal and actinorhizal symbioses in non-legumes. Method Microbiol 24:417–432

Dawson JO, Kowalski DG, Dart PJ (1989) Variation with soil depth, topographic position and host species in the capacity of soils from an Australian locale to nodulate *Casuarina* and *Allocasuarina* seedlings. Plant Soil 118:1–11

Dell B, Malajczuk N, Bougher NL, Thomson G (1994) Development and function of *Pisolithus* and *Scleroderma* ectomycorrhizas formed in-vivo with *Allocasuarina, Casuarina* and eucalyptus. Mycorrhiza 5:129–138

Diem HG (1996) Mycorrhizae of antinorhizal plants. Acta Bot Gallica 143:581–592

Diem HG, Dommergues YR (1985) *In vitro* production of specialized reproductive torulose hyphae by *Frankia* strain ORS 021001 isolated from *Casuarina junghuhniana* root nodules. Plant Soil 87:17–29

Diem HG, Dommergues YR (1990) Current and potential uses and management of Casuarinaceae in the tropics and subtropics. In: Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic, New York, pp 317–342

Diem HG, Duhoux E, Zaid H, Arahou M (2000) Cluster roots in Casuarinaceae: role and relationship to soils nutrient factors. Ann Bot 85:929–936

Dommergues YR (1995) Nitrogen fixation by trees in relation to soil nitrogen economy. Nutr Cycl Agroecosyst 42:215–230

Dommergues YR, Subba-Rao NS (2000) Introduction of N_2-fixing trees in non-N_2-fixing tropical plantations. In: Subba-Rao NS, Dommergues YR (eds) Microbial interactions in agriculture and forestry, Science Publishers, Enfield, N.H., USA, pp 131–154

Duponnois R, Diedhiou S, Chotte JL, Sy MO (2003) Relative importance of the endomycorrhizal and (or) ectomycorrhizal associations in *Allocasuarina* and *Casuarina* genera. Can J Microbiol 49:281–287

El-Giahmi AA, Nicolson, TH, Daft MJ (1976) Endomycorrhizal fungi from Libyan soils. Mycol Res 67:164–170

Fleming AI, Wittenberg JB, Wittenber BA, Dudman WF, Appleby CA (1987) The purification, characterization and ligand-binding kinetics of hemoglobins from root nodules of the non-leguminous *Casuarina glauca-Frankia* symbiosis. Biochem Biophys Acta 911:209–220

Forrester DI, Bauhus J, Cowie AL, Vanclay JK (2006) Mixed-species plantations of *Eucalyptus* with nitrogen-fixing trees: a review. For Ecol Manage 233:211–230

Franche C, Laplaze L, Duhoux E, Bogusz D (1998) Actinorhizal symbioses: recent advances in plant molecular and genetic transformation studies. Crit Rev Plant Sci 17:1–28

Frank, AB (1888) Üeber die physiologische Bedeutung der Mycorhiza. Ber Dtsch Bot Ges 6:248–269

Gardner IA (1986) Mycorrhizae of actinorhizal plants. MIRCEN J Appl Microbiol Biotechnol 2:147–160

Gauthier DL, Diem HG, Dommergues Y (1983) Preliminary results of research on *Frankia* and endomycorrhizae associated with *Casuarina equisetifolia*. In: Midgley SJ, Turnbull JW, Johnson RD (eds), Casuarina ecology, management and utilisation. CSIRO Forest Research, Melbourne, Australia, pp 211–217

Gherbi H, Duhoux E, Franche C, Pawlowski K, Nassar A, Berry AM, Bogusz D (1997) Cloning of a full-length symbiotic hemoglobin cDNA and in situ localization of the corresponding mRNA in *Casuarina glauca* root nodule. Physiol Plant 99:608–616

Goel VL, Behl HM (2005) Growth and productivity assessment of *Casuarina glauca* Sieb. ex. Spreng on sodic soil sites. Bioresource Technol 96:1399–1404

He XH, Critchley C, Ng H, Bledsoe CS (2004) Reciprocal N ($^{15}NH_4^+$ or $^{15}NO_3^-$) transfer between non-N_2-fixing *Eucalyptus maculate* and N_2-fixing *Casuarina cunninghamiana* linked by the ectomycorrhizal fungus *Pisolithus* sp. New Phytol 163:629–640

He XH, Critchley C, Ng H, Bledsoe CS (2005) Nodulated N_2-fixing *Casuarina cunninghamiana* is the sink for net N transfer from non-N_2-fixing *Eucalyptus maculate* via an ectomycorrhizal fungus *Pisolithus* sp. supplied as ammonium nitrate. New Phytol 167:897–912

Jasper DA, Robson AD, Abbott LK (1979) Phosphorus and the formation of vesicular-arbuscular mycorrhizas. Soil Biol Biochem 11:501–505

Khan AG (1993) Occurrence and importance of mycorrhizae in aquatic trees of New South Wales, Australia. Mycorrhiza 3:31–38

Kohls SJ, Baker DD (1989) effects of substrate nitrate concentration on symbiotic nodule formation in actinorhizal plants. Plant Soil 118:171–179

Kumar A (1981) Problems and prospects of establishing a plantation forestry for *Casuarina*, cashew and coconut in the coastal belt of India. Riv Agric Subtropic Tropic 75:317–323

Lambers H, Shane MW, Cramer MD, Pearse SJ, Veneklaas EJ (2006) Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. Ann Bot 98:693–713

Lancelle SA, Torrey JG, Hepler PK, Callaham DA (1985) Ultrastructure of freeze-substituted *Frankia* strain HFPCc13, the actinomycete isolated from root nodules of *Casuarina cunninghamiana*. Protoplasma 127:64–72

Laplaze L, Duhoux E, Franche C, Frutz T, Svistoonoff S, Bisseling T, Bogusz D, Pawlowski K (2000) *Casuarina glauca* prenodule cells display the same differentiation as the corresponding nodule cells. Mol Plant Microbe Interact 13:107–112

Lechevalier MP, Lechevalier HA (1990) Systematics, isolation and culture of *Frankia*. In: Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic, New York, pp 35–60

Lugo AE (2004) The outcome of alien tree invasions in Puerto Rico. Front Ecol Environ 2:265–273

Mariotti A, Sougoufara B, Dommergues YR (1992) Estimation of nitrogen-fixation using the natural abundance method in a plantation of *Casuarina equisetifolia* (Forst). Soil Biol Biochem 24:647–653

Mark GL, Hooker JE, Hahn A, Wheeler CT (1999) In vitro culture of arbuscular mycorrhizal fungus and *Frankia* for inoculation of micropropagated *Casuarina equisetifolia* L. Can J Bot 77:1391–1397

Midgley SJ, Turnbull JW, Johnston RD (1983) *Casuarina*: ecology, management and utilisation. CSIRO Forest Research, Melbourne, Australia

National Academy of Sciences (1980) Firewood crops. Shrub and tree species for energy production. Report of an Ad Hoc Panel of the Advisory Committee on Technology Innovation, Board on Science and Technology for International Development, Commission on International Relations, Washington DC, USA

National Research Council (1984) Casuarinas: nitrogen-fixing trees for adverse sites. Office of International Affairs, Washington DC, USA

Newcomb W, Wood SM (1987) Morphogenesis and structure of *Frankia* (Actinomycetales): the microsymbiont of nitrogen, fixing actinorhizal root nodules. Int Rev Cytol 109:1–88

Obertello M, Sy MO, Laplaze L, Santi C, Svistoonoff S, Auguy F, Bogusz D, Franche C (2003) Actinorhizal nitrogen fixing nodules: infection process, molecular biology and genomics. Afr J Biotechnol 2:528–538

Olson DF, Petteys EQP (1974) *Casuarina*. In: Schopmeyer CS (ed) Seeds of woody plants in the United States. Agriculture Handbook 450, U.S. Department of Agriculture, Washington, DC. pp 278–280

Osundina MA (1998) Nodulation and growth of mycorrhizal *Casuarina equisetifolia* J. R. and G. First in response to flooding. Biol Fert Soils 26:95–99

Parrotta JA (1999) Productivity, nutrient cycling, and succession in single- and mixed-species plantations of *Casuarina equisetifolia, Eucalyptus robusta*, and *Leucaena leucocephala* in Puerto Rico. For Ecol Manage 124:45–77

Parrotta JA, Baker DD, Fried M (1999) Changes in dinitrogen fixation in maturing stands of *Casuarina equisetifolia* and *Leucaena leucocephala*. Can J For Res 26:1684–1691

Parsons R, Silvester WB, Harris S, Gruijters WTM, Bullivant J (1987) *Frankia* vesicles provide inducible and absolute oxygen protection for nitrogenase. Plant Physiol 83:728–731

Pinyopusarerk K, Turnbull JW, Midgley SJ (1996) Recent Casuarina Research and Development: Proceedings of the Third International Casuarina Workshop, Da Nang, Viet Nam, 4–7 Mar 1996. CSIRO Forestry and Forest Products, Canberra, Australia pp

Rajendran K, Devaraj P (2004) Biomass and nutrient distribution and their return of *Casuarina equisetifolia* inoculated with biofertilizers in farmland. Biomass Bioenergy 26:235–249

Rajendran K, Sugavanam V, Devaraj P (2004) Effect of microbial inoculation on quality seedling production of *Casuarina equisetifolia*. J Trop For Sci 15:82–96

Reddell P (1990) Increasing productivity in plantings of *Casuarina* by inoculation with *Frankia*. In: El-Lakany, MH, Turnbull JW, Brewbaker JL (eds) Advances in *Casuarina* research and utilization, Desert Development Center, Cairo, pp 133–140

Reddell P, Bowen GD, Robson M (1986) Nodulation of *Casuarinaceae* in relation to host species and soil properties. Aust J Bot 34:435–444

Reddell P, Rosbrook PA, Bowen GD, Gwaze D (1988) Growth responses in *Casuarina cunninghamiana* plantings to inoculation with *Frankia*. Plant Soil 108:79–86

Reddell P, Yun Y, Shipton WA (1997) Cluster roots and mycorrhizae in *Casuarina cunninghamiana*: their occurrence and formation in relation to phosphorus supply. Aust J Bot 45:41–51

Resh SC, Binkley D, Parrotta JA (2002) Greater soil carbon sequestration under nitrogen-fixing trees compared with *Eucalyptus* species. Ecosystems 5:217–231

Rockwood DL, Huffman JB, Conde LF (1983) Potential of *Casuarina* spp for biomass production in Florida. Silvicultura 8:376–377

Rose S (1980) Mycorrhizal associations of some actinomycete nodulated nitrogen fixing plants. Can J Bot 58:1449–1454

Sanginga N, Danso SKA, Bowen GD (1989) Nodulation and growth-response of allocasuarina and casuarina species to phosphorus fertilization. Plant Soil 118:125–132

Sanginga N, Bowen GD, Danso SKA (1990) Genetic variability in symbiotic nitrogen fixation within and between provenances of two *Casuarina* species using the ^{15}N-labeling methods. Soil Bio Biochem 22:539–547

Santi C, von Groll U, Ribeiro A, Chiurazzi M, Auguy F, Bogusz D, Franche C, Pawlowski K (2003) Comprison of nodule induction in legume and actinorhizal symbioses: the induction of actinorhizal nodules does not involve ENOD40. Mol Plant Microbe Interact 16:808–816

Schubert KR (1986) Products of biological nitrogen fixation in higher plants: synthesis, transport, and metabolism. Annu Rev Plant Physiol 37:530–574

Schwencke J, Carú M (2001) Advances in actinorhizal symbiosis: host plant-*Frankia* interactions, biology, and applications in arid land reclamation. A review. Arid Land Res Manage 15:285–327

Sempavalan J, Wheeler CT, Hooker JE (1995) Lack of competition between *Frankia* and *Glomus* for infection and colonization of roots of *Casuarina equisetifolia* (L). New Phytol 130:429–436

Shah SK, Shah RP, Xu LH, Aryal UK (2006) Biofertilizers: an alternative source of nutrients for sustainable production of tree crops. J Sustain Agric 29:85–95

Sidhu OP, Behl HM, Gupta ML, Janardhanan KK (1990) Occurrence of vesicular-arbuscular mycorrhiza in *Casuarina equisetifolia* L. Curr Sci 59:422–423

Silvester WB, Harris SL, Tjepkema JD (1990) Oxygen regulation and haemoglobin. In: Schwintzer CR, Tjepkema JD (eds) The biology of *Frankia* and actinorhizal plants. Academic, New York, pp 157–177

Simonet P, Navarro E, Rouvier C, Reddell P, Zimpfer J, Dommergues Y, Bardin R, Combarro P, Hamelin J, Domenach AM, Gourbiere F, Prin Y, Dawson JO, Normand P. (1999) Co-evolution between *Frankia* populations and host plants in the family Casuarinaceae and consequent patterns of global dispersal. Environ Microbiol 1:525–533

Singh CS, Kapoor A, Rana JPS (1998) Occurrence of ecto- and endo-mycorrhiza with the roots of *Casuarina equisetifolia* in various soil types. Indian Forester 124:561–564

Smith SE, Read DJ (1997) Mycorrhizal symbiosis (2nd edn). Academic, San Diego, USA

Sougoufara B, Diem HG, Dommergues YR (1989) Response of field-grown *Casuarina equisetifolia* to inoculation with *Frankia* strain ORS 021001 entrapped in alginate beads. Plant Soil 118:133–137

Sprent JI, Parsons R (2000) Nitrogen fixation in legume and non-legume trees. Field Crop Res 65:183–196

Sprent JI, Sprent P (1990) Nitrogen fixing organisms: pure and applied aspects. Chapman & Hall, London

Subba-Rao NS, Rodriguez-Barrueco C (1995) *Casuarinas*. Science Publishers, Lebanon, N.H.

Swensen SM (1996) The evolution of actinorhizal symbioses: evidence for multiple origins of the symbiotic association. Am J Bot 83:1503–1521

Theodorou C, Reddell P (1991) *In vitro* synthesis of ectomycorrhizas on *Casuarinaceae* with range of mycorrhizal fungi. New Phytol 118:279–288

Thoen D, Sougoufara B, Dommergues Y (1990) Invitro mycorrhization of *Casuarina* and *Allocasuarina* species by *Pisolithus* isolates. Can J Bot 68:2537–2542

Torrey JG (1976) Initiation and development of root nodules of *Casuarina (Casuarinaceae)*. Am J Bot 63:335–345

Torrey JG (1983) Root development and root nodulation in *Casuarina*. In: Midgley SJ, Turnbull JW, Johnson RD (eds) *Casuarina* ecology, management and utilisation. CSIRO Forest Research, Melbourne, Australia, pp 180–192

Torrey JG, Callaham D (1982) Structural features of the vesicle of *Frankia* sp. CpI1 in culture. Can J Microbiol 28:749–757

Vasanthakrishna M, Bagyaraj DJ (1993). Selection of efficient VA mycorrhizal fungi for inoculating *Casuarina equisetifolia*. Arid Soil Res Rehabil 7:377–380

Vasanthakrishna M, Bagyaraj DJ, Nirmalnath PJ (1994a) Responses of *Casuarina equisetifolia* to inoculation with *Glomus fasciculatum* and/or *Frankia*. For Ecol Manag 68:399–402

Vasanthakrishna M, Bagyaraj DJ, Muthanna MB (1994b) VA mycorrhizal fungi associated with *Casuarina equisetifolia*. Microbiol Res 149:23–25

Vasanthakrishna M, Bagyaraj DJ, Nirmalnath PJ (1995) Selection of efficient VA mycorrhizal fungi for *Casuarina equisetifolia*–second screening. New For 9:157–162

Wall LG (2000) The actinorhizal symbiosis. J Plant Growth Reg 19:167–182

Wang B, Qiu YL (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. Mycorrhiza 16:299–363

Warcup JH (1980) Ecotomycorrhizal associations of Australian indigenous plants. New Phytol 85:531–535

Wheeler CT, Tilak M, Scrimgeour CM, Hooker JE, Handley LL (2000) Effects of symbiosis with *Frankia* and arbuscular mycorrhizal fungus on the natural abundance of ^{15}N in four species of *Casuarina*. J Exp Bot 51:287–297

Wilson KL, Johnson LAS (1989) Casuarinaceae. In: George AS (ed) Flora of Australia, vol 3 – Hamamelidales to Casuarinales. Australian Government Publishing Service, Canberra, Australia, pp 1–219

Wilson KL, Berendsohn WG (2005) IOPI global plant checklist: Casuarinaceae. International Organization for Plant Information (IOPI). Royal Botanic Gardens, Sydney NSW 2000, Australia (http://plantnet.rbgsyd.nsw.gov.au/iopi/iopihome.htm)

Yang Y (1995) The effect of phosphorus on nodule formation and function in the *Casuarina-Frankia* symbiosis. Plant Soil 118:125–132

Zaïd EH, Arahou M, Diem HG, Morabet R (2003) Is Fe deficiency rather than P deficiency the cause of cluster root formation in *Casuarina* species? Plant Soil 248:229–235

Zimpfer JF, Kaelke CM, Smyth CA, Hahn D, Dawson JO (2003) *Frankia* inoculation, soil biota, and host tissue amendment influence *Casuarina* nodulation capacity of a tropical soil. Plant Soil 254:1–10

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